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TEXTBOOK OF BIOCHEMISTRY
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Textbook of

BIOCHEMISTRY

Textbook of

BIOCHEMISTRY

by BENJAMIN HARROW, Ph.D.
Professor of Chemistry, City College
College of the City of New York

FOURTH EDITION,
ILLUSTRATED

W. B. SAUNDERS COMPANY
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To Sources of Inspiration:

C. H., M. H. L., M. T. L., P. H. L., P. L., F. M.

PREFACE TO THE FOURTH EDITION

BIOCHEMISTRY is advancing rapidly and extensively, necessitating frequent revisions of a text such as this one. The very welcome reception given to previous editions has made the present revision all the more imperative.

In addition to many minor improvements throughout the book, the main changes may be summarized as follows:

1. A chapter on the properties of solutions has been added. Many will want to use this chapter in connection with Chapter 4 on proteins. This added chapter has been put in the appendix (p. 552) for two reasons: so as not to disturb a certain logical continuity of the chapters; and because the subject matter treated is elementary in nature and is meant more in the form of "refresher" material for students who have not had inorganic or physical chemistry for some time.

2. Included also in the appendix are several discussions of a character possibly too advanced for the general student. These include proofs of structures and several syntheses; for example, evidence for the structure of cholesterol (p. 549), and syntheses of glutathione (p. 563), thiamine (p. 568) and two of the sex hormones (pages 572 and 573).

3. More or less extensive changes have been made in the following sections of the book:

Emphasis on the cyclic structure of the sugars (chapter 2).

Revision of the section on starch (p. 23).

Deletion of parts of Chapter 4 (proteins) and their incorporation in the chapter on solutions (p. 552).

Association of nucleoproteins with chromosomes and genes (p. 78).

A more complete classification of the proteolytic enzymes (pages 92 and 93).

The processing of foods (p. 128).

Social problems connected with food consumption (p. 129).

Food in Europe during World War II (p. 131).

Antagonistic action of analogous compounds, (for example, p. 162).

Additions to niacin (p.162).

Addition to pyridoxine (p. 165).

Antibiotins (p. 168).

Relation of *p*-aminobenzoic acid to sulfanilamide (p. 169).

More on gastric analysis (p. 221), gallstones (p. 229) and jaundice (p. 230), and, in general, more clinical material throughout the book.

Origin of plasma proteins (p. 265), deficiency of plasma proteins (p. 265) and clinical use of blood plasma (p. 266).

Malaria and quinine (p. 282).

Mode of action of sulfonamides (p. 285)

Antibiotic substances (p. 288, 294), including a detailed discussion of penicillin (p. 289).

Streptomycin (p. 295).

More complete summaries of conversion of glycogen to lactic acid (p. 324) and of the oxidation of carbohydrate (p. 327).

The connection of thiamine with carbohydrate metabolism (p. 328).

Multiple oxidation of fatty acids (p. 340).

Enlarged section on the metabolism of cholesterol (p. 346).

More on deamination (p. 353).

The clinical importance of the nitrogenous constituents of the blood (p. 381).

Dehydration (p. 442).

Myosin and adenosinetriphosphatase (p 446)

Teeth (p. 453).

Origin of urinary ammonia (p. 471).

Analogues of thyroxine (p. 482).

Thiourea and thiouracil and the thyroid (p. 482).

Alloxan diabetes (p. 496).

Glutamic acid and the brain (p. 536).

More on acetylcholine (p. 537).

Food requirements of the Army (p. 567).

My thanks are due to many colleagues, more particularly to Professor P. M. Apfelbaum and Drs. E. Borek and H. Wagreich (City College), Professor M. Karshan (Columbia) and Professor I. Greenwald (New York University).

My thanks are also due the publishers, W. B. Saunders Company, for help generously offered.

BENJAMIN HARROW

New York City

PREFACE

IN THIS book the story of biochemistry is told in the form of closely knit chapters, written, I hope, in simple and clear English. The book includes the latest developments in the field It covers—and, I believe, more than covers—the usual requirements of courses in biochemistry offered to medical, dental, agricultural, and general college students.

In the attempt to ensure accuracy, various colleagues throughout the country were asked to read and criticize portions of the manuscript. They did so most willingly, and their suggestions have been of great value. They are, of course, in no way responsible for the book with its probable shortcomings.

Something should be said about the use of references. The references are placed at the end of each chapter. They have been selected with some care, and their particular virtues are pointed out. The emphasis has been placed upon reviews rather than on original papers, though the latter are by no means excluded. In fact, individual papers have been selected with a view to training the student in the experimental method of approach. Knowing the difficulties which the average student encounteres with a foreign language, preference has been given to articles in English. The review articles, appearing, for example, in Physiological Reviews, or in the Annual Review of Biochemistry, contain all the needed references to original sources.

I am deeply indebted to the following gentlemen who have read and freely criticized the various chapters of the book: Dr. Morton Anson (Rockefeller Institute); Prof. P. M. Apfelbaum (College of the City of New York); Dr. Max Bergmann (Rockefeller Institute); Dr. Ernest Borek (College of the City of New York); Prof. Henry Borsook (California Institute of Technology); Prof. R. K. Cannan (New York University); Prof. C. F. Cori (Washington University); Prof. C. A. Elvehjem (University of Wisconsin); Prof. J. H. Ferguson (University of Michigan); Dr. J. S. Fruton (Rockefeller Institute); Prof. Harold Himwich (Albany Medical College); Prof. Maxwell Karshan (Columbia University); Dr. Moses Kunitz (Rockefeller Institute); Dr. R. M. Herriott (Rockefeller Institute); Prof. H. B. Lewis (University of Michigan); Prof. J. M. Luck (Stanford University); Prof. E. V. McCollum (Johns Hopkins University); Dr. Abraham Mazur (College of the City of New York); Prof. Rudolf Schoenheimer (Columbia University); Dr. C. P. Sherwin; Prof. W. M. Sperry (Columbia University); Dr. G. C. H. Stone (College of the City of New York); Dr. R. S. Tipson (Rockefeller Institute); Dr. Harry Wagreich (College of the City of New York).

My thanks are also due the publishers, W. B. Saunders Company, for help in many directions.

BENJAMIN HARROW

New York City

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ABBREVIATIONS

(For general reviews consult the *Ann. Rev. Biochem.*, *Ann. Rev. Physiol.*, *Physiol. Rev.*, and *Harvey Lectures*. References to original papers are given. For further references see *Chemical Abstracts*.)

Adv. Enzym. = Advances in Enzymology
Am. J. Bot. = American Journal of Botany
Am. J. Diseases Children = American Journal of Diseases of Children
Ann. = Annalen der Chemie
Ann. Rev. Biochem. = Annual Review of Biochemistry
Ann. Rev. Physiol. = Annual Review of Physiology
Arch. Biochem. = Archives of Biochemistry
Arch. Internal Med. = Archives of Internal Medicine
Ber. = Berichte der deutschen chemischen Gesellschaft
Biochem. J. = Biochemical Journal
Biochem. Z. = Biochemische Zeitschrift
Biol. Rev. Cambridge Phil. Soc. = Biological Reviews of the Cambridge Philosophical Society
Bull. N. Y. Acad. Med. = Bulletin of the N. Y. Academy of Medicine
Chem. Rev. = Chemical Reviews
Ind. Eng. Chem. = Industrial and Engineering Chemistry
J. Am. Chem. Soc. = Journal of the American Chemical Society
J. Am. Med. Assoc. = Journal of the American Medical Association
J. Biol. Chem. = Journal of Biological Chemistry
J. Chem. Educ. = Journal of Chemical Education
J. Gen. Physiol. = Journal of General Physiology
J. Nutrition = Journal of Nutrition
J. Phys. Chem. = Journal of Physical Chemistry
J. Physiol. = Journal of Physiology
J. Soc. Chem. Ind. = Journal of the Society of Chemical Industry
Nutr. Rev. = Nutritional Reviews
Physiol. Rev. = Physiological Reviews
Proc. Nat. Acad. Sci. U. S. = Proceedings of the National Academy of Sciences of the United States of America
Proc. R. S. (London), Series B = Proceedings of the Royal Society (London)
Proc. Soc. Exp. Biol. Med. = Proceedings of the Society for Experimental Biology and Medicine
Skand. Arch. Physiol. = Skandinavisches Archiv für Physiologie
Symp. Quant. Biol. = Symposia on Quantitative Biology
Z. physiol. Chem. = Zeitschrift für physiologische Chemie

CHAPTER 1

INTRODUCTION

WITHOUT attempting a definition of biochemistry, we may say that it deals, among other things, with the chemical processes which go on in living matter. Chemical processes common to plants come under the heading of "plant biochemistry"; and this phase of the subject is barely touched upon in the present volume. A short chapter on photosynthesis (Chap. 9) merely scratches the surface. For further information, see, for example, Tottingham, *Plant Biochemistry.*

In this volume we devote ourselves to animal biochemistry, and when the word "biochemistry" (or "physiological chemistry") alone is used, we usually mean "animal biochemistry."

While it is difficult to trace origins, one is tempted to speak of Lavoisier (1743–1794) not only as the father of modern chemistry, but also as the father of biochemistry; for he was certainly one of the first, if not the first, to appreciate the true nature of respiration. His classical researches into oxidation, and the central rôle played by oxygen in the process, led him to investigate "burning" in the body; and he came to the conclusion that oxygen is consumed in the reaction, that carbon dioxide is eliminated, and that heat is evolved. He also realized that the temperature of the body is the result of the oxidation of foodstuffs. Later, in the hands of Voit, Pettenkofer and Rubner in Germany, and Atwater and Benedict in this country, animal calorimetry (Chap. 19) became a science in the modern sense.

Liebig (1803–1873) and Wöhler (1800–1882), two organic chemists, had much to do with the further development of the subject; for their researches led them, from time to time, to analyze material of vegetable and animal origin. Liebig arrived at the conclusion that "the nutritive materials of all green plants are inorganic substances"; and Wöhler's dramatic synthesis of urea, the principal end-product of nitrogenous metabolism in the body, did much to destroy the notion that animal products were endowed with a "vitalism" which made them fundamentally different from "lifeless" substances. The work of Chevreul (1786–1889) on the chemical constitution of fats, and later the researches of Kossel and Emil Fischer on proteins, and of Emil Fischer on carbohydrates, gradually led to an understanding of the chemical composition of foods and the chemical composition of the cell.

Nor must the influence of the illustrious Pasteur (1822–1895) be overlooked. His extensive researches into the nature of fermentation led Buchner (1860–1917) to our modern conception of enzymes (Chap. 6), the cellular catalysts which are responsible for much of the activities within the body. Further, Pasteur's work on fermentation was a prelude to much activity in the field of muscle metabolism (Chap. 16),

and, even more recently, to work dealing with the metabolism in the brain (Chap. 25).

Researches by such pioneers as Arrhenius, van't Hoff, and Ostwald on electrolytic dissociation and osmotic pressure, led physical chemists, as well as organic chemists, to turn their attention to biological phenomena. The results were very fruitful; among others, Sörensen developed our concept of *p*H (Chap. 4), Loeb examined the colloidal behavior of proteins (Chap. 4), and L. J. Henderson and Van Slyke developed their ideas regarding "body neutrality" (Chap. 15).

Side by side, physiologists and clinicians were contributing much of great value to the biochemist in such fields as digestion (Chap. 10), absorption (Chap. 12), blood (Chap. 13), and metabolism (Chaps. 16, 17, 18, 20).

Nor can we overlook the impetus to further work given by the founding, in 1879, of the first journal devoted to biochemistry, the *Zeitschrift für physiologische Chemie.* In 1906 three other journals were started: the *Journal of Biological Chemistry* in this country; the *Biochemical Journal* in England; and the *Biochemische Zeitschrift* in Germany. To this day, the bulk of the important literature in biochemistry still appears in these four journals.

Present-day activity in biochemistry is very varied; but some of the most sensational successes in recent times have been in the fields of vitamins, hormones, enzymes and nucleoproteins. A number of vitamins and hormones have been not only isolated but even synthesized; and some of them have turned out to be relatively simple substances (Chaps. 8, 24). Enzymes are probably all proteins, and therefore very complex (Chap. 6). Not the least interesting discovery is that several vitamins are probably "mother substances" of enzymes active in metabolism (Chap. 19). And chromosomes and some viruses, at least, are being more and more associated with nucleoproteins (Chap. 5).

These activities must be coupled with the activities in the field of chemotherapy, with its sulfanilamides and the amazing penicillin (Chap. 14).

At the beginning of the present century there was already an active laboratory of biochemistry in this country. Chittenden had been its founder at Yale. One of his pupils, Mendel, succeeded him; and another, Gies, became professor of the subject at Columbia. Folin was appointed to a chair of biochemistry at Harvard in 1907. The guiding spirits in the medical schools quickly recognized the importance of the subject, and chairs of biochemistry sprang up all over the country.

To such an extent has biochemistry developed, here and abroad, that a substantial portion of *Chemical Abstracts* is devoted to abstracts of biochemical articles. In the attempt to summarize a field which has become so extensive and diversified, the *Annual Review of Biochemistry* was founded in 1932.

* * * *

The chemical composition of the cell is discussed in the opening chapters. The substances which have been identified include carbohy-

drates, lipids, proteins, and enzymes. Water and inorganic salts, also present, are more appropriately considered in the section devoted to metabolism. Hormones, also present, are discussed towards the end of the book in connection with coordinating mechanisms of the body.

The chapters dealing with synthesis in the plant kingdom, foods, and vitamins are a preliminary to a discussion of digestion and absorption. The digested materials are carried by the blood to the various cells of the body. A discussion of this "carrier," the blood, together with methods by which foods are oxidized, leads to chapters on blood, respiration, various phases of metabolism, and the mechanism of biological oxidation. The elimination of substances from the body leads to a discussion of urine. The nervous system and the glands of internal secretion (which manufacture hormones) represent the coordinating links of the body; and the concluding chapters are devoted to them.

The dramatic clinical results obtained with sulfanilamide and penicillin have suggested Chap. 14 (immunochemistry and chemotherapy).

References

Chittenden, *The Development of Physiological Chemistry in the United States* (1930).

Von Meyer, *History of Chemistry* (1891).

Lieben, *Geschichte der physiologischen Chemie* (1935).

CHAPTER 2

CARBOHYDRATES

THE carbohydrates include substances which are constituents of the cell, compounds which are important foods, and products which find industrial application. They include polyhydroxyaldehydes and polyhydroxyketones, and compounds which can be converted into such aldehydes or ketones by hydrolysis.

The simplest of these compounds is glycolaldehyde, $CHO.CH_2OH$, which exhibits many of the properties of carbohydrates; but the latter are all optically active, containing asymmetric carbon atoms; which makes glyceraldehyde, $CHO.CHOH.CH_2OH$, the more logical "mother" substance.

Classification.—The more important of these carbohydrates may be classified as follows:

Monosaccharides.	Pentoses, $C_5H_{10}O_5$ (ribose, xylose, arabinose, etc.) Hexoses, $C_6H_{12}O_6$ (glucose, mannose, galactose, fructose, sorbose, etc.)
Disaccharides.	$C_{12}H_{22}O_{11}$ (sucrose, lactose, maltose, isomaltose, etc.)
*Trisaccharides.**	$C_{18}H_{32}O_{16}$ (raffinose, etc.)
Polysaccharides.	$(C_6H_{10}O_5)_x$ (starch, glycogen, dextrin, cellulose, gum, mucilage, inulin, etc.)

The monosaccharides cannot be hydrolyzed into simpler sugars. By the use of the appropriate acid or enzyme the higher saccharides can be hydrolyzed:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow C_6H_{12}O_6 + C_6H_{12}O_6$$
Sucrose $\rightarrow$ Glucose + Fructose
Maltose $\rightarrow$ Glucose + Glucose
Lactose $\rightarrow$ Glucose + Galactose

$$C_{18}H_{32}O_{16} + 2H_2O \rightarrow C_6H_{12}O_6 + C_6H_{12}O_6 + C_6H_{12}O_6$$
Raffinose $\rightarrow$ Glucose + Fructose + Galactose

A number of the polysaccharides, upon complete hydrolysis, yield glucose as the end-product (for example, glycogen, starch, dextrin, cellulose); some yield fructose (for example, inulin); some yield galactose (for example, certain gums).

MONOSACCHARIDES

Structure of Glucose.—The somewhat exceptional position occupied by glucose in carbohydrate metabolism (Chap. 16) and the impossibility, owing to limitations of space, of discussing the structure of

* The term *oligosaccharides* is also used for compounds made up of 2 to 5 molecules of monosaccharides. Above this number we deal with polysaccharides.

each sugar individually, make it desirable that we describe glucose in some detail. Much of this discussion holds for other sugars.

A qualitative analysis of a purified sample of glucose shows the presence of the elements carbon and hydrogen; a quantitative analysis will also reveal the presence of oxygen. The elements are in such proportion to one another that the formula (CH_2O) can be assigned to the compound. A molecular weight determination (by the freezing point depression method, for example) reveals that the formula assigned should be $(CH_2O)_6$, or $C_6H_{12}O_6$.

Glucose forms an oxime with hydroxylamine (p. 20), an osazone with phenylhydrazine (p. 20), and reduces Benedict's solution (p. 18); all of these reactions point to the presence of a $-\overset{|}{\underset{|}{C}}=O$ group, and this group may represent an aldehyde or a ketone.

Glucose forms a pentaacetyl derivative with acetic anhydride, indicating the presence of five free hydroxyl groups. It is reduced by means of sodium amalgam to an alcohol, hexahydroxyhexane, $CH_2OH.(CHOH)_4.CH_2OH$, which is sorbitol; and the latter compound, when treated with hydrogen iodide, is converted to a derivative of *normal* hexane, $CH_3.(CH_2)_3.CHI.CH_3$. With hydrogen cyanide glucose forms an addition compound which when hydrolyzed gives a straight-chain seven-carbon acid. All these facts point to a straight-chain compound, with the $-\overset{|}{\underset{|}{C}}=O$ at one end (a compound containing the aldehydic group).

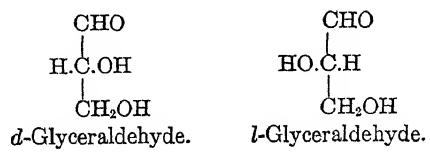
1. CHO
2. CHOH
3. CHOH
4. CHOH
5. CHOH
6. CH_2OH

Isomers of Glucose.—An examination of the formula reveals that the compound has four asymmetric carbon atoms (at positions 2, 3, 4, and 5), each carbon being attached to four different atoms or groups of atoms. For example, the carbon at position 2 may be shown thus:

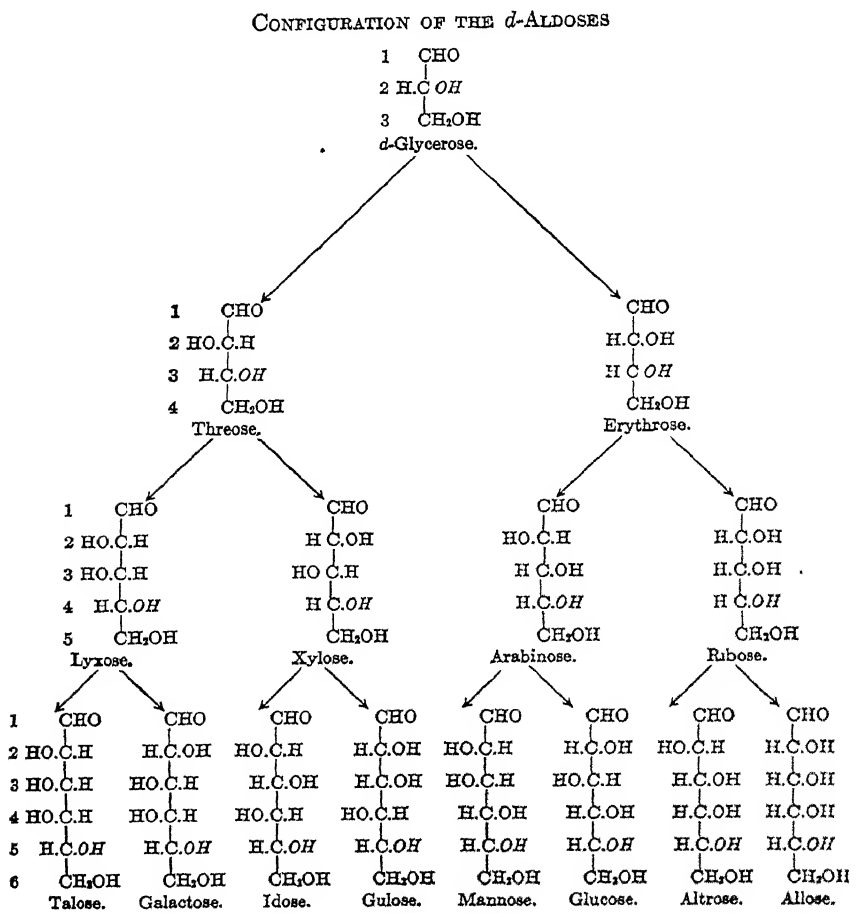
$$\begin{array}{c} R \\ | \\ H-C-OH \\ | \\ R^1 \end{array}$$

where R stands for the CHO group and R^1 for everything below carbon 2. According to van't Hoff, the number of possible isomers is given by the formula $I = 2^n$, where n represents the number of asymmetric carbon atoms. Since glucose has four such asymmetric carbon atoms, the number of isomers equals 16 (2^4).

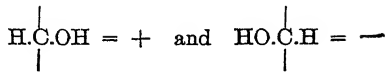
The Spatial Arrangement of the Isomers of Glucose.—The rigorous proof for the stereochemical configuration of each isomer of glucose is beyond the scope of this book. Taylor and also Whitmore (see references at the end of the chapter) give accounts of this phase of the work. All that can be said at this point is that the isomers are traced back to some simple compound the constitution of which is beyond question. We may, for example, regard these isomers as being derived from glyceraldehyde:



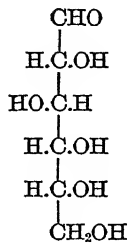
From each isomer of this aldehyde there are derived eight isomers of glucose. For example, the accompanying chart shows the derivation and configuration of the eight *dextro*-aldohexoses:



Using the notation that

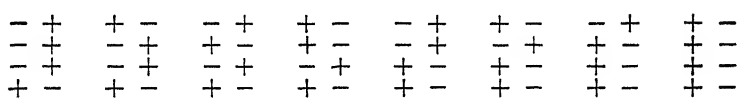


then *d*-glucose,



can be represented in shorthand form as

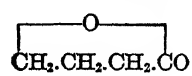
Here the two end-carbon combinations are ignored in this notation. We can represent the sixteen possible isomers as follows:



The first of each pair represents the *d*-series (related to *d*-glucose and *d*-glyceraldehyde), and the second, the *l*-series.

In this *d*-series, the hydroxyl group next to the primary alcohol group is written to the right; whereas the reverse is true with the *l*-series.

The Cyclic Structure for Glucose.—However, the above formula for glucose still does not explain all the facts. To begin with, glucose, which is pictured as an aldehyde, falls somewhat short of certain common aldehydic properties. For example, glucose fails to give a Schiff's test (the formation of a reddish violet color with magenta solution which has been decolorized with SO_2); nor does it form a stable addition compound with sodium bisulfite. Hydroxy acids of the γ- or δ-variety, similar in general structure to glucose, form lactones very readily; and these are cyclic in structure; for example, γ-hydroxybutyric acid, $CH_2OH.CH_2.CH_2.COOH$, is changed over to γ-butyrolactone,



But even more important is the problem of **mutarotation** or "change of rotation." A freshly crystallized sample of glucose has a specific rotation (in water) of $+111°$ ($[\alpha]_D + 111°$); upon standing, this changes to $+52°$. Since the specific rotation of any compound is, as a rule, characteristic of the optically active compound in question (just as melting and boiling points are usually characteristic criteria), a change in rotation suggests a change in the structure of the substance.

This appears all the more probable when it is shown that the compound with the rotation +111° (now known as α-glucose) can be dissolved in boiling pyridine and crystallized from this solvent to give an isomer with the specific rotation of +19° (now known as β-glucose), which, upon standing, also changes slowly to +52°. The β-form is the more stable one at temperatures around 100° C.

Another fact has to be recorded now. When an aldehyde is treated with methyl alcohol (using an acid to catalyze the reaction), an acetal is formed.

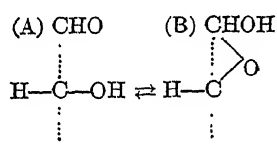
$$R.CHO + 2CH_3OH \rightarrow R.CH(OCH_3)_2 + H_2O$$

Here two molecules of methyl alcohol are used for one molecule of the aldehyde. When, however, glucose is treated similarly, the sugar combines with but *one* molecule of methyl alcohol:

$$C_6H_{12}O_6 + CH_3OH \rightarrow C_7H_{14}O_6 + H_2O$$

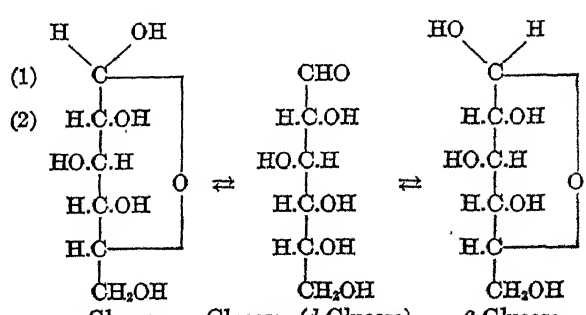
The methyl glucoside which is formed can be resolved into two modifications: an α-methyl glucoside (rotation +159°) and a β-methyl glucoside (rotation −34°). The enzyme maltase hydrolyzes the former initially to α-glucose, and the enzyme emulsin hydrolyzes the latter initially to β-glucose (followed by mutarotation in each case).

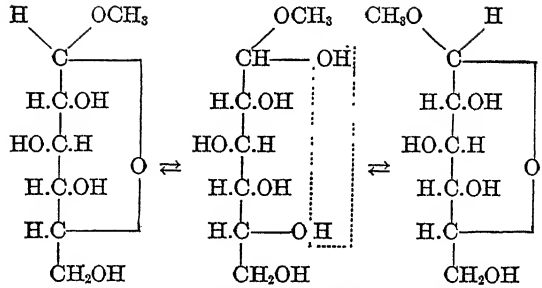
The two modifications each of *d*-glucose and the corresponding methyl glucoside suggest the presence of an additional asymmetric carbon atom in the molecule, which can be shown if we assume a cyclic structure:



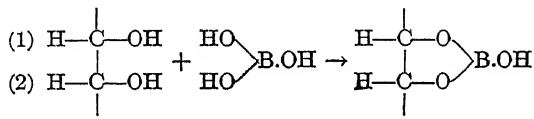
Here the carbon atom at (A), part of an ordinary aldehydic group, has been converted into an asymmetric carbon atom (B); so that now in glucose we have the possibility not of 16 (= 2^4), but of 32 (= 2^5) isomeric aldohexoses.

In the accompanying chart the structures and relationships of the compounds just discussed are given (the evidence for the oxygen bridge in the 1:5 position will be taken up presently).



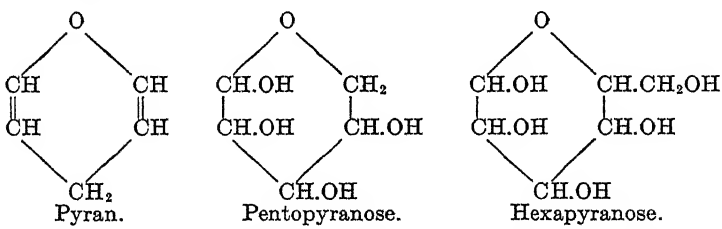


It might be pointed out that there are reasons for writing the H and OH positions at carbon (1) in α- and β-glucose as shown. For example, α-glucose combines very readily with boric acid:



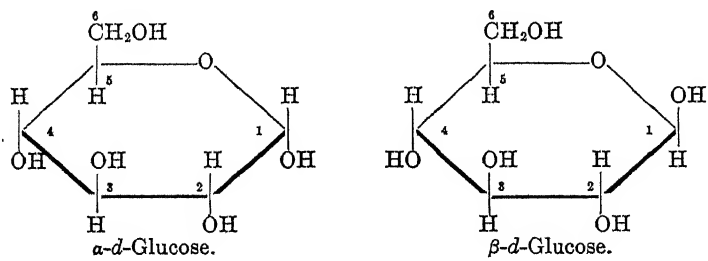
suggesting that the two OH groups in (1) and (2) are on the right. The β-form, however, does not combine so readily with boric acid until mutarotation has given some α-form. The names α- and β- are based on Hudson's rule: in sugars related to *d*-glucose, the rotation of the β-form subtracted from the rotation of the α-form gives a positive difference.

Assuming, for the time being, a 1:5 oxygen bridge, glucose may be pictured as a derivative of a pyran, the 5-carbon sugar form for which would be a pentopyranose, and the 6-carbon sugar a hexopyranose:



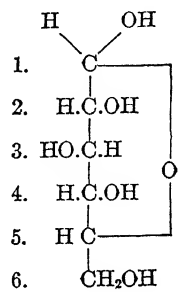
One such hexopyranose, glucose, can therefore be called glucopyranose.

Haworth shows this model in perspective, with the H and the OH groups above or below the plane of the ring:



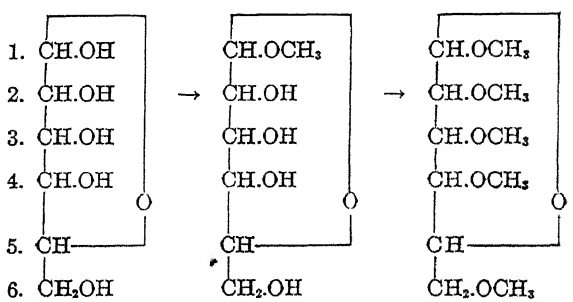
The ring is at right angles to the plane of the paper. The thin bonds of the ring are behind the plane of the paper, and the thick bonds in front of it. (H and OH attached to carbon atoms 1, 2, 3, 4 which are on the *right* side of the straight-chain formula are here on the *bottom* side.)

Methylation Studies. Evidence for the 1 : 5 Oxygen Bridge.—It has been assumed so far that the oxygen is attached to carbons 1 and 5.

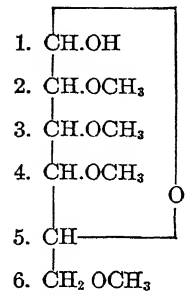


There are obviously several other possible attachments. As a matter of fact, but two forms have been isolated: the 1.5 and the 1:4. Of these, the 1:5 is the more stable and the commoner form.

After the methylglucoside has been formed (with $CH_3OH + HCl$), the product is next treated with dimethyl sulfate. This results in complete methylation of the OH groups. Without using the stereochemical formulas, and still assuming the 1:5 oxygen bridge, the reactions are as follows:

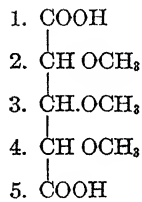


Now it should be pointed out that the glucosidic methoxyl group can be easily hydrolyzed, whereas the other methoxyls cannot—behaving, indeed, as true ethers. If, then, we hydrolyze the completely methylated compound, we get tetramethyl glucopyranose; and the latter,



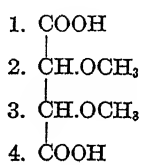
Tetramethyl glucopyranose.

upon oxidation with nitric acid, yields a trimethyl derivative of glutaric acid:

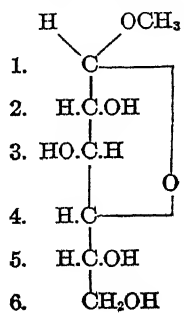


which indicates that the oxygen is attached to carbon atom 5 in the glucoside.

"Gamma" or "Active" Glucose.—Ordinarily, in the formation of methyl glucosides, hot alcoholic HCl is used. This forms the 1:5 oxygen bridge linkage, as we have already seen. If, however, cold alcoholic HCl is used, a far less stable compound is formed; and this differs from the more stable form by having a 1·4 linkage. Complete methylation and final oxidation with nitric acid lead to the production of a succinic acid derivative:

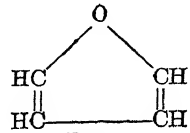


showing that this methylglucoside has the structure



or a 1:4 oxygen bridge.

Just as the parent substance of the 1:5 sugars is pyran, so the parent compound of the 1:4 sugars is furan:



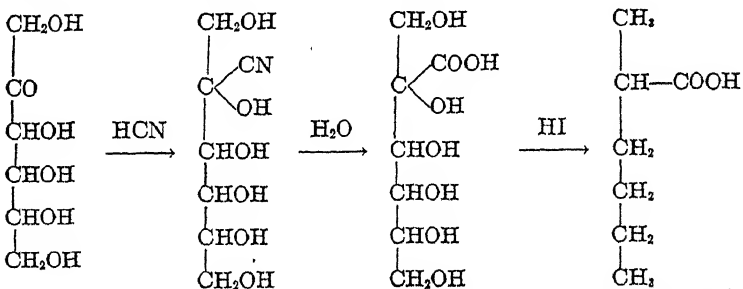
Furan

and the sugars derived from it are furanose sugars.

These furan sugars were formerly referred to as "gamma" or "active" sugars; and it is believed by some that such "gamma" sugars are important "intermediates" in the oxidation of carbohydrates in the body.

The Structure of Fructose.—From the physiological point of view, glucose, fructose, and galactose are the three important hexoses.

Fructose, a naturally occurring sugar, is levorotatory; but despite this fact, the sugar is known as *d*-fructose, because it is related, structurally, to *d*-glucose.* It has the same molecular formula as glucose ($C_6H_{12}O_6$), forms a pentaacetyl derivative and shows the properties of a carbonyl (C=O) compound. When fructose is treated with HCN it forms an addition compound, which upon hydrolysis and subsequent reduction with HI gives methylbutylacetic acid:



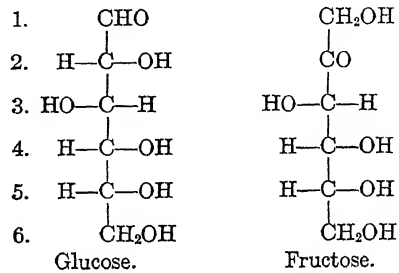
Fructose. Methylbutylacetic acid.

and this means that fructose is a ketone and not an aldehyde. (Under similar conditions, glucose yields a 7-carbon straight-chain and not a branched-chain compound.)

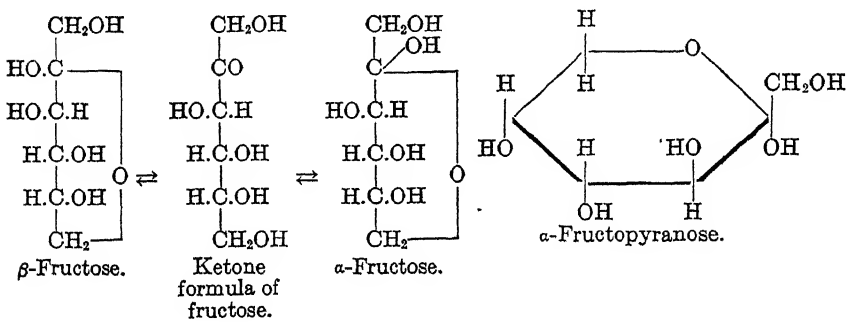
The oxidation of fructose (with HgO and $Ba(OH)_2$) yields glycollic acid, $CH_2OH.COOH$, and trihydroxybutyric acid, $CH_2OH.(CHOH)_2.COOH$, indicating a splitting of the compound between carbons 2 and 3.

* The symbols *d*- and *l*- refer to *configuration*, whereas dextro (+) and levo (−) refer to *sign of rotation*. *d* (+) glucose is related structurally to *d* (−) fructose, for both give the same osazone (p. 20), but glucose rotates the plane of polarized light to the right, and fructose, to the left. The mirror images of these compounds are referred to as the *l*-series, quite regardless of whether the compounds are dextro- or levorotatory. (See footnote, p. 50.)

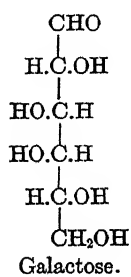
Both glucose and fructose give the same osazone (p. 20); and this, as we shall see (p. 21), points to the fact that in fructose, the groups at 3, 4, and 5 must be the same as those in glucose:



Like glucose, fructose exhibits the property of mutarotation; and, again like glucose, it normally forms a 2:6 oxygen bridge linkage, giving a fructopyranose. These structures may be summarized as follows:



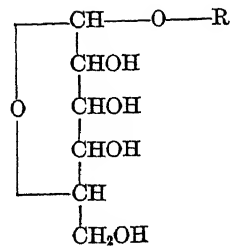
Galactose.—Proof of the formula for *d*-galactose



is too involved to be given here (see Taylor, p. 293, etc.). Mutarotation (β- and α-forms) with pyranose formations occurs here too.

DISACCHARIDES

The disaccharides may be regarded as glycosides of the form:

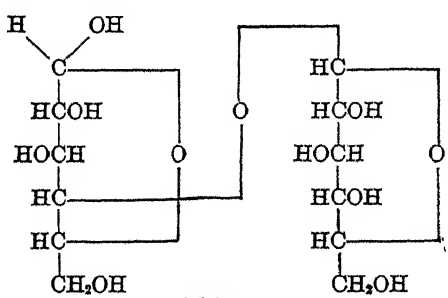


where *R* is replaced by a monosaccharide which (1) has a potential free aldehydic group (as in maltose and lactose), or (2) which has no such reducing group (see sucrose, p. 15).

Where *R* is replaced by CH_3 we have the methylglycoside which has already been discussed (p. 8). In nature, important glycosides are known where *R* is a nonsugar, often a complicated group. For example, phlorizin (also spelled *phloridzin* and *phlorhizin*), found in the bark of Rosaceae, is a combination of glucose and phloretin; amygdalin, present in the seed of the bitter almond, is a combination of glucose and mandelonitrile; digitonin (p. 40), found in the leaves and seeds of digitalis, is a combination of glucose, galactose, and digitogenin. In the cardiac glycosides (found in certain plants and possessing a characteristic action on the heart) the "R" is represented by a sterol (Chap. 3). (According to Armstrong, *glycoside* is the general name for this group of compounds, irrespective of the sugar present; a *glucoside* is the more specific name for those glycosides which contain glucose as the sugar constituent.)

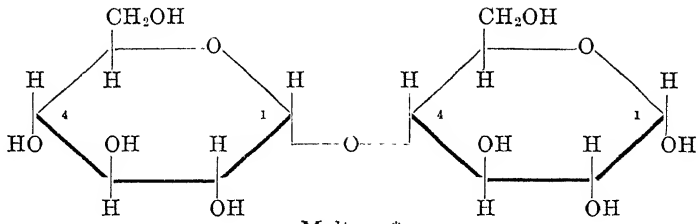
As has already been pointed out, maltose, lactose, and sucrose, the three common disaccharides, are easily hydrolyzed to monosaccharides. The structures of these disaccharides, in turn, are dependent upon the monosaccharides they yield.

Maltose yields two molecules of glucose when hydrolyzed. Upon methylation and subsequent hydrolysis, we get 2,3,4,6-tetramethylglucose and 2,3,6-trimethylglucose. While these results lead to two possible structures, the following has been selected as more in accord with the facts:



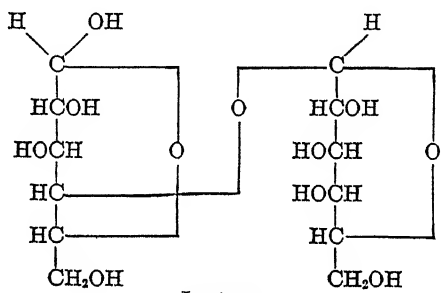
Maltose.

or, using the Haworth model,



Maltose.*

Lactose yields glucose and galactose on hydrolysis. When the lactose is methylated and then hydrolyzed, we get 2,3,6-trimethyl-glucose and 2,3,4,6-tetramethyl galactose; this leads eventually to the formula:†

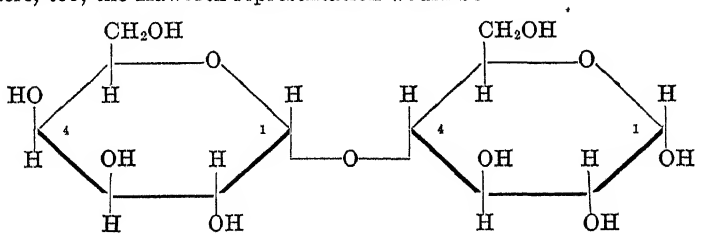


Lactose.

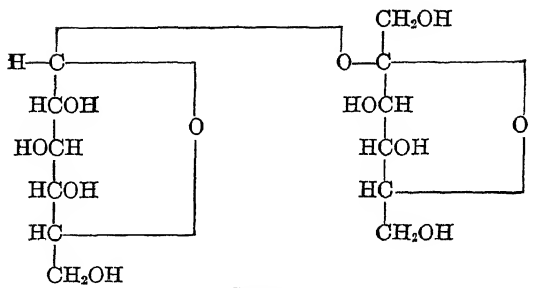
Sucrose, unlike maltose and lactose, does not reduce Benedict's solution (p. 19). We say it is a nonreducing sugar. When hydrolyzed, it yields glucose and fructose. Methylation and subsequent hydrolysis convert sucrose into a tetramethyl glucose and a tetramethyl fructose, leading to the formula:

* This is really α-maltose (or α-glucoside-α-glucose), where the active (aldehyde) group is at position 1 on the right side of the formula. There is also a β-maltose (or α-glucoside-β-glucose), in which the H and OH in position 1, on the right side of the formula, is reversed. **Cellobiose,** obtained from cotton cellulose as its acetyl derivative, which is also made up of two glucose molecules, differs from maltose in being β-glucoside-α-glucose.

† Here, too, the Haworth representation would be



Showing a β-galactose unit on the left and an α-glucose unit on the right. This is α-lactose. The β-lactose is shown by reversing the HOH in position 1 of the glucose unit; giving β-galactose and β-glucose units.



Sucrose.

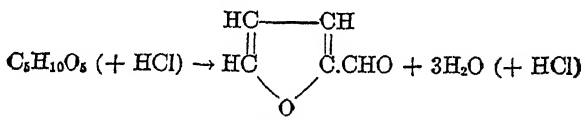
Sucrose has a pyranose structure for the glucose part, and a furanose structure for the fructose part. It is, therefore, a glucosido-fructofuranoside.*

Sucrose has been synthesized from glucose-1-phosphate (p. 322) and fructose by using the enzyme phosphorylase obtained from the bacterium, *Pseudomonas saccharophila.*

SOME GENERAL PROPERTIES OF THE MONO- AND DISACCHARIDES

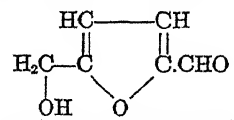
These substances are crystalline, soluble in water, and moderately soluble in dilute alcohol. They are practically insoluble in absolute alcohol, ether, and the usual organic solvents.

The Action of Nonoxidizing Acids.—Certain acids (like HCl) hydrolyze the disaccharides. (They also hydrolyze tri- and polysaccharides) On the monosaccharides themselves their action is negligible if the acid is dilute. In higher concentrations, and at the boiling point, such acids act on monosaccharides largely by dehydrating them, forming furfural derivatives. The pentoses form furfural.



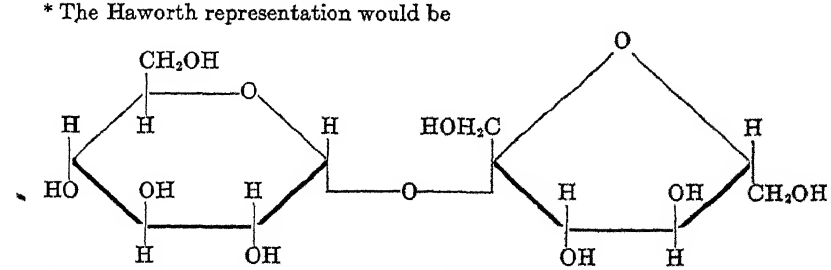
Furfural.

The hexoses yield varying quantities of hydroxymethylfurfural.



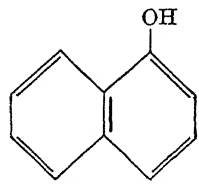
Hydroxymethylfurfural.

* The Haworth representation would be



represented by an α-glucose and a β-fructose unit.

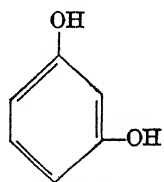
The formation of such furfurals is the basis for a number of tests. One of the commonest of such tests is the *Molisch test:* the sugar mixed with α-naphthol



α-Naphthol.

and concentrated sulfuric acid gives a violet color. It is presumed that the concentrated sulfuric acid acts as a dehydrating agent, acting on the sugar to form furfural derivatives, which then combine with α-naphthol to form colored products of uncertain constitution. This is a very general test for carbohydrates.

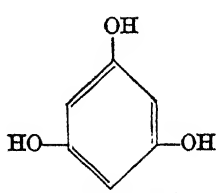
A more specific test is the *Seliwanoff test:* the action of resorcinol



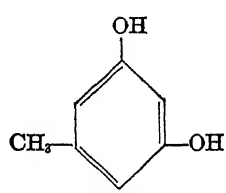
Resorcinol.

and HCl on the sugar. Here a red color is developed rapidly in the presence of a ketose sugar, usually fructose. The explanation of the test lies in the formation, first, of hydroxymethylfurfural and the condensation of this substance with resorcinol to form the colored product or products.

One or two tests for pentoses, *Tollens' phloroglucinol test* and *Tollens' orcinol test,* are based on the formation of similar intermediate furfural products and ultimate condensations to yield colored substances.



Phloroglucinol.

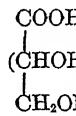


Orcinol.

Oxidation.—Alkaline copper solutions readily oxidize the disaccharides (with the exception of sucrose) and the monosaccharides. The oxidation products are numerous, and not all of them have been identified. But, from a practical point of view, this reaction is important because with the oxidation of the sugar there is a simultaneous reduction of the cupric to a cuprous compound: usually insoluble, red cuprous oxide, which can be identified easily. The best-known reagents

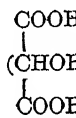
are *Fehling's solution* [an alkaline (NaOH) copper sulfate solution, with potassium sodium tartrate to keep the cupric oxide in solution] and *Benedict's solution* [an alkaline (Na_2CO_3) copper sulfate solution in the presence of sodium citrate]. An acid copper solution (copper acetate in acetic acid), known as *Barfoed's reagent*, is often used, though not always satisfactorily, to distinguish mono- from disaccharides. Under identical conditions, the monosaccharides are more rapidly oxidized.

With bromine water as the oxidizing agent, glucose forms a gluconic acid,



Gluconic acid. *

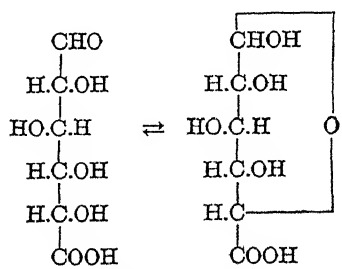
and with nitric acid, saccharic acid is formed.



Saccharic acid.

With galactose, nitric acid yields an isomer, *mucic acid*, which, unlike saccharic, is highly insoluble. This mucic acid test is used to identify galactose (or lactose, which first hydrolyzes to form galactose as one of the products).

When saccharic acid is heated, it forms the corresponding lactone, which can be reduced to **glucuronic acid** with sodium amalgam.



Glucuronic acid.

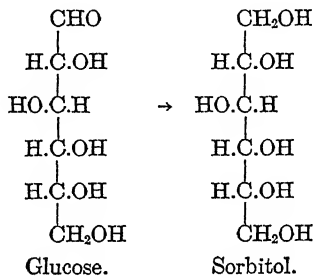
This oxidation product of glucose, which *in vitro* has to be obtained in a rather indirect way from the sugar, is a physiologically important substance, since it acts as a detoxifying agent. It combines with poisonous substances† which find their way into the body, thereby

* Gluconic acid may also be obtained from glucose by the action of various microorganisms (*e.g.*, Aspergillus niger). The calcium gluconate is a form of calcium salt used in therapeutics.

† Sometimes glucuronic acid combines with substances which can hardly be considered as poisonous. For example, the female hormones are eliminated by the body as glucuronides (see p. 510).

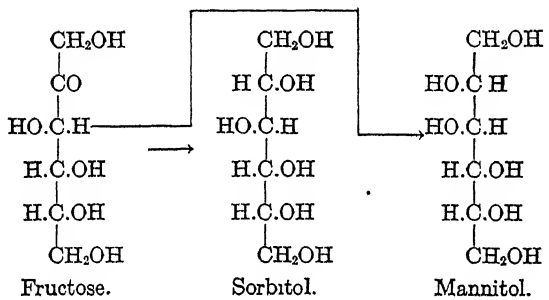
making them nontoxic, the coupled products then being eliminated (see Chap. 11).

Reduction.—Reduction (with sodium amalgam) converts the monosaccharides to the corresponding alcohols. Glucose, for example, gives sorbitol.



Glucose. Sorbitol.

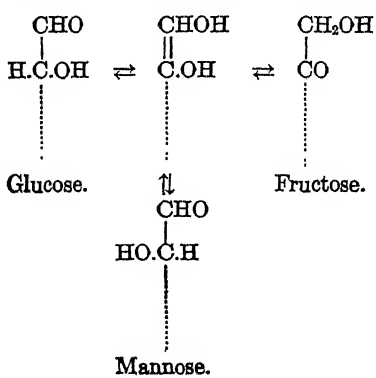
Fructose yields a mixture of sorbitol and mannitol, since the carbon of the ketonic group now becomes asymmetric.



Fructose. Sorbitol. Mannitol.

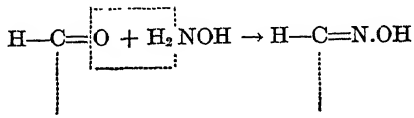
These alcohols are used in culture media for the differentiation of certain organisms.

The Action of Weak Alkali.—Using a saturated solution of barium hydroxide, and allowing the mixture to stand for some time, we can transform glucose into an equilibrium mixture of glucose, mannose, and fructose. The same holds true if instead of glucose we begin with fructose or mannose. This has been explained on the assumption that a reactive "intermediate" (enol) compound is formed:

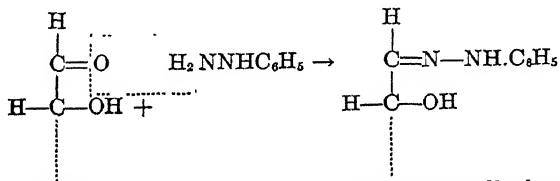


These tautomeric changes, known as the *Lobry de Bruyn transformation*, are of interest to biochemists in helping to explain how some monosaccharides are converted into glycogen in the liver (p. 312).

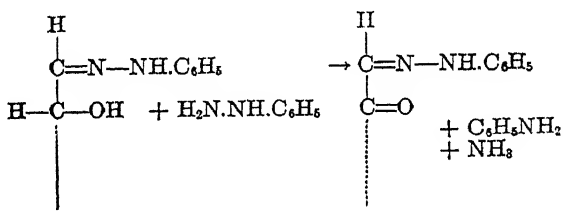
Osazones.—The monosaccharides and the disaccharides (with the exception of sucrose) combine with hydroxylamine to form oximes:



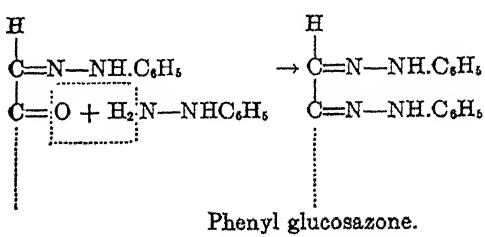
but from a practical standpoint, the more important reaction is the formation of osazones with phenylhydrazine, $C_6H_5NHNH_2$. At first, one molecule of the sugar combines with one molecule of the phenylhydrazine to form a hydrazone:



Next, in the presence of an excess of phenylhydrazine, another molecule of this reagent reacts with the sugar,

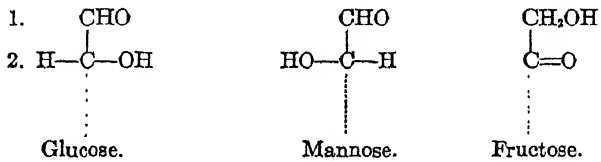


converting the secondary alcohol group of the hydrazone into a ketone; and finally, a third molecule of the reagent enters the reaction giving an osazone:



These osazones are yellow, insoluble crystalline compounds, fairly characteristic in form (and other properties) for each individual sugar; so that the osazone test becomes an important one for purposes of identification.

Glucose, mannose, and fructose give the same osazone. This must mean that such sugars differ only in the first 2 carbon loadings:



With fructose, the first molecule of the phenylhydrazine reacts with the carbonyl group; the second molecule is involved in the reaction which oxidizes the primary alcoholic group (next to the carbonyl group) to an aldehyde; and the third molecule of the reagent reacts with this aldehydic group to form the osazone.

Phenylhydrazine was first prepared by Emil Fischer. He, also, was the first to use this reagent in the field of carbohydrates.

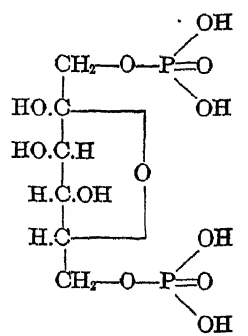
Fermentation.—The three hexoses which are fermented by yeast are glucose, mannose and fructose; and they are fermented only when they are the naturally occurring *d*-forms (which means when they are structurally related to *d*-glyceraldehyde). The simple equation representing the reaction is:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

If a disaccharide such as sucrose is used, then this sugar is first hydrolyzed by an enzyme in yeast into a mixture of "invert sugar" (glucose + fructose), which then undergoes fermentation.

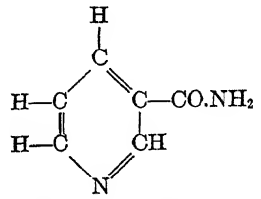
The enzyme system in yeast responsible for the fermentation includes *zymase.*

It is now known that the activity of the zymase is conditioned by the presence of a number of substances, among them phosphates and a cozymase. The phosphate is necessary for a preliminary union with hexose to form one or more hexosephosphates. One such combination which has been isolated is fructofuranose-1:6-diphosphoric acid:



Another substance which plays a vital part in the fermentation process is spoken of as a "cozymase," or "coenzyme I," a substance stable to heat, which can be obtained from an autolyzed yeast extract by boiling it and filtering the product (a process which destroys the

zymase) This cozymase has been isolated and has been shown to be a rather complex combination of the purine adenine (p. 85), the 5-carbon sugar ribose (p. 6), phosphoric acid and the amide of nicotinic acid:



Nicotinic acid amide.

This cozymase is chemically closely related to the coenzyme found in muscle (Chaps. 16 and 19).

The cozymase of yeast and the coenzyme of muscle are examples of "coenzymes" found in cells which, in a general way, differ from the enzymes proper. Enzymes are heat-labile, nondialyzable compounds of high molecular weight—probably proteins—produced by the living cells and active as catalysts in chemical reactions (see Chap. 6). Coenzymes are also produced by the living organisms, but they are not so complex chemically, and are relatively heat-stable and dialyzable.*

DESCRIPTIONS OF SOME MONO- AND DISACCHARIDES

Glucose, $C_6H_{12}O_6$ (for structure, etc., see p. 6), also called **dextrose** and **grape sugar,** occurs with fructose in sweet fruits. It is the normal sugar present in blood. In diabetes the amount in the blood increases, and very often, a considerable quantity of this sugar appears in the urine (p. 473). Its preparation from cornstarch by acid hydrolysis is a commercial process. Glucose is surpassed in sweetness only by fructose and sucrose.

Fructose, $C_6H_{12}O_6$ (for structure, etc., see p. 6), also called **levulose** and **fruit sugar,** is present in sweet fruits, together with glucose. It is now obtained on a commercial scale by the hydrolysis of inulin (p. 26), a polysaccharide found in the Jerusalem artichoke.

Galactose, $C_6H_{12}O_6$ (for structure, see p. 6), is obtained when agar (an Asiatic sea-weed) or lactose is hydrolyzed. It is found, in combination, in nerve tissue (Chaps. 3 and 25).

Mannose, $C_6H_{12}O_6$, an aldohexose (for structure, see p. 6), occurs in combination in mannans (found, for example, in the ivory nut from which buttons are made). While glucose, galactose, and fructose are common foodstuffs, mannose plays but a minor rôle.

Maltose, $C_{12}H_{22}O_{11}$ (for structure, see p. 14), or **malt sugar,** is obtained when starch is hydrolyzed by an enzyme (diastase or amylase) found in sprouting barley or malt. It is also a product formed when the enzyme in saliva (ptyalin) acts on starch (p. 23). When hydrolyzed (by acid or by the enzyme maltase of the small intestine), glucose is formed. For each molecule of maltose we obtain two molecules of glucose.

* For further details see p. 99.

Lactose, $C_{12}H_{22}O_{11}$ (for structure, see p. 15), or **milk sugar,** is the sugar present in milk. It is sometimes found in the urine of women during lactation. When hydrolyzed (by acid or by the enzyme lactase of the small intestine) a mixture of glucose and galactose is obtained.

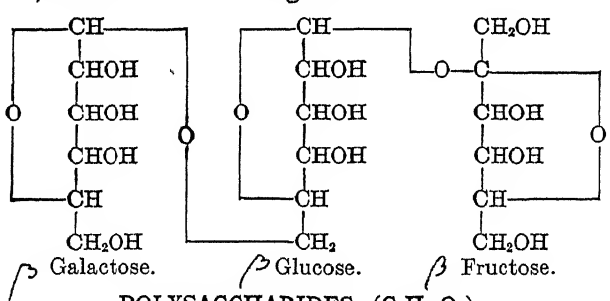
Sucrose, $C_{12}H_{22}O_{11}$ (for structure, see p. 16), also called **cane sugar** or **saccharose,** occurs in abundance in the sugar cane and the sugar beet, and is the sugar commonly used for sweetening purposes. When hydrolyzed (by acid, by the enzyme invertase of yeast, or by the enzyme sucrase of the intestine) a mixture of glucose and fructose is obtained. This mixture is known as "invert sugar" because the sucrose is dextrorotatory, whereas the product obtained is levorotatory. The fructose molecule turns the plane of polarized light to the left more than does the glucose molecule to the right.

PENTOSES, $C_5H_{10}O_5$

The pentoses, the 5-carbon sugars, are more common in the plant than in the animal kingdom. They are found in abundance in gums and pentosans (p. 26). The structural formulas for some of them, and their relations to the hexoses, are given on p. 6. **Arabinose** (obtained from gum arabic), **xylose** (obtained from wood gums) and **ribose,** found in animal tissues and cell nuclei, are common examples.

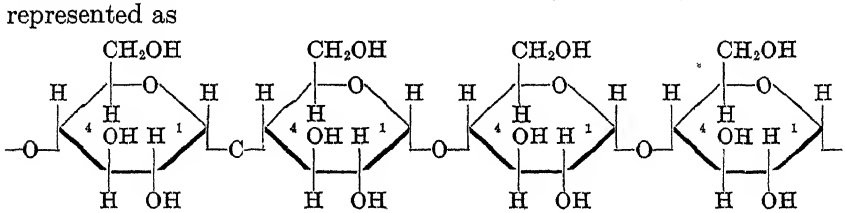
TRISACCHARIDES, $C_{18}H_{32}O_{16}$

The best-known of the trisaccharides is **raffinose,** a vegetable product found in cottonseed and in the molasses obtained from beet sugar. It can be partially hydrolyzed to **melibiose,** a disaccharide; this is a galactosido-α-glucose. It is ultimately hydrolyzed to galactose, glucose, and fructose; and its structure is given as

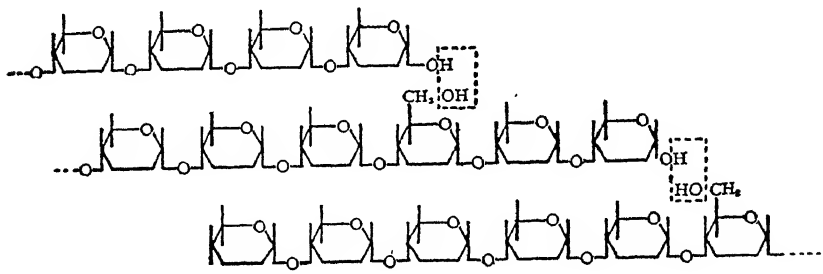


POLYSACCHARIDES, $(C_6H_{10}O_5)_x$

Starch, which occurs abundantly in grains, tubers and fruits, is largely the source of carbohydrates for man. It consists of two types of molecules: linear or non-branched polymer of glucose (amylose) and a branched polymer of glucose (amylopectin). The amylose may be represented as



which shows non-branched chains of glucopyranose units joined by the first and fourth carbon atoms. The amylopectin, the branched type, shows joining of branches at 1,6-glucosidic linkages (compare the numbering on p. 9 for formula for glucose):



The average chain length (glucose units) in amylose ranges from 200 to 500. This amylose is slightly soluble in water, forms a slightly viscous solution, and is hydrolyzed to maltose by the enzyme, β-amylase. (The amylases obtained from saliva, pancreatic juice and germinating seeds, and the diastase obtained from malt hydrolyze starch, as a whole, to maltose.) With iodine amylose gives a more powerful blue color than does amylopectin.

Amylopectin itself constitutes some 80 per cent of many starches It is highly viscous and is incompletely hydrolyzed by β-amylase. Phosphoric acid and fatty acids are often found as part of the molecule of amylopectin. Ignoring the phosphoric acid and the fatty acids, starch, or its two constituents, when hydrolyzed completely (by acid), yields glucose and nothing else. Like the other polysaccharides, and unlike the mono- and disaccharides, starch does not dialyze.

When boiled with water, the starch swells and forms a paste. A soluble starch can be obtained by a preliminary treatment of starch with cold dilute hydrochloric acid. The hydrolysis of starch by ptyalin or amylase (the enzyme in saliva) results in the production of a number of dextrins and, finally, maltose. Like the other polysaccharides, starch does not reduce Benedict's solution nor form an osazone; so that free ketonic or aldehydic groups are absent.

A form of synthetic starch can be obtained by the action of an enzyme, phosphorylase, obtained from an extract of muscle on glucose-1-phosphate (p. 91). The same result may be obtained by using the potato tuber as the source of the phosphorylase. This synthetic starch resembles amylose in being poorly soluble in water, in yielding an intense color with iodine, and in being hydrolyzed completely to maltose by β-amylase. Oddly enough, when the source of phosphorylase is heart or liver, the polysaccharide which is obtained resembles glycogen (p. 91) rather than starch. The product, for example, is soluble in water and gives a reddish-brown color with iodine.

Dextrins, such as erythrodextrin (which gives a reddish color with iodine) and achroodextrin (which does not give a color with iodine),

are formed in the early stages of the hydrolysis of starch. They have the general formula $(C_6H_{10}O_5)_x$.

Glycogen, or animal starch, is found in liver and muscle. It is soluble in water (unlike starch) and gives a red color with iodine (starch gives a blue color). It is hydrolyzed *in vivo* to glucose; and when hydrolyzed *in vitro* by acid, glucose is again the only product. Like starch, it fails to reduce Benedict's solution or to form an osazone. Haworth is of the opinion that, in structure, glycogen resembles starch, the difference residing in the number of glucose residues in the chain composing the molecule. The glycogen molecule, as a rule, contains chains of 12 glucose units, whereas starch has chains of 24 to 30 units. As a rule, glycogen, like starch, contains phosphorus in some organic combination.

A substance resembling glycogen has been found in lower plants (fungi, yeasts and bacteria); and glycogen, in addition to starch, has been isolated from the seed of the sweet corn. The isolation of glycogen from corn is important because it has always been assumed that this polysaccharide is a typical animal product, whereas now it is shown to be present in one of the higher plants as well.

It has been shown by Cori that animal tissues contain an enzyme, phosphorylase, which hydrolyzes glycogen (in the presence of phosphate) into glucose-1-phosphate (p. 322). Under suitable conditions, the phosphorylase can act on glucose-1-phosphate to reconvert it into glycogen (p. 322).*

The molecule of glycogen is also made up of glucose molecules in branched chains.

Cellulose, the constituent of the cell wall of plants, is a highly insoluble substance. It can be dissolved in Schweitzer's reagent (ammoniacal cupric hydroxide), in an acid (HCl) solution of zinc chloride and in a solution of sodium hydroxide and carbon disulfide, the last forming *viscose,* from which rayon is made. It is hydrolyzed with difficulty, the product formed being glucose. Unlike starch, glycogen and the dextrins, which are readily digested, cellulose passes through the human digestive tract without being attacked by any of the digestive enzymes. Its structure probably resembles that of starch; but there is still much uncertainty in this field. In any case, it is believed that whereas starch (and glycogen) consist of α-glucosidic chains, cellulose consists of β-glucosidic combinations.†

Inulin, present in the Jerusalem artichoke, is soluble in hot water, gives a negative iodine test, and yields fructose on hydrolysis. It has

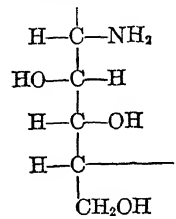
* Compare under starch, p. 24.

† Cellulose is very widely distributed. Cotton, linen and wood are rich in this substance. By changing the physical form of cotton (there are several methods available), rayon, known for a time as artificial silk, is produced. Cellulose acetate forms the basis for motion picture films and shatter-proof glass. From cellulose nitrate a number of important industrial products are also obtained; for example, guncotton, celluloid, collodion, lacquers, etc.

More than 80 per cent of the rayon manufactured is made by the viscose process. Spruce pulp or cotton linters are soaked in caustic soda, treated with carbon disulfide (to form "viscose") and forced through fine holes using an acid bath, thereby forming filaments of regenerated cellulose (rayon). These filaments can then be twisted to form threads.

been suggested that the molecule of this polysaccharide consists of 30 fructose units and has a molecular weight of about 5000.

Chitin, found in the skeletal material of the Insecta and Crustacea, yields glucosamine on hydrolysis:



Glucosamine.

Glucosamine is also obtained when the mucin of saliva (Chap. 10) and the mucoids of connective tissues are hydrolyzed.

The **pentosans** are polysaccharides which yield pentoses on hydrolysis. Examples of pentosans are found in gum arabic, from which arabinose is obtained, and in oat hulls and corn cobs, which yield xylose. The **galactans,** such as agar-agar, another common plant product, yield galactose on hydrolysis. The **pectins,** present in apples, lemons, etc., and which form fruit gels with sugar, give on hydrolysis some galactose and arabinose; but galacturonic acid—resembling glucuronic acid—(p. 18) is the principal product.

References

Many of the following books and articles contain abundant references to the original literature.

Tipson and Stiller's chapter on carbohydrates in Harrow and Sherwin's *Textbook of Biochemistry* (1935) is an excellent review of the subject.

The chapters on carbohydrates by Wolfrom, Raymond and Heuser in Gilman's *Organic Chemistry* (1943), pages 1352, 1605 and 1664 are strongly recommended

Among organic texts, the one by Taylor, *Reactions and Symbols of Carbon Compounds* (1930), and that by Whitmore, *Organic Chemistry* (1937), have unusually detailed chapters on the carbohydrates. See, also, Fuson and Snyder, *Organic Chemistry* (1942) and Fieser and Fieser, *Organic Chemistry* (1944)

Haworth, one of the foremost authorities on the chemistry of the carbohydrates, is the author of *The Constitution of the Sugars* (1929).

Reviews of yearly progress are summarized in the *Ann. Rev. Biochem.*, of which the first volume appeared in 1932. See, for example, Hassid, **13,** 59 (1944); Hurd, **14,** 91 (1945).

A chemical synthesis of lactose is given by Haskins, Hann and Hudson in the *J. Am. Chem. Soc.*, **64,** 1852 (1942). An enzymic synthesis of sucrose is described by Hassid, Doudoroff and Barker, *Ibid.*, **66,** 1416 (1944).

A fine review of Fischer's original method for determining the configuration of glucose is given by Hudson, *J. Chem. Educ.*, **18,** 353 (1941).

A standard book on alcoholic fermentation is by Harden (1932).

The separation of starch into its two constituents is described by Pacsu and Mullen, *J. Am. Chem. Soc.*, **63,** 1168 (1941) and by McCready and Hassid, *Ibid.*, **65,** 1154 (1943).

For a study of the nature of the starch-iodine complex, see Rundle, Foster and Baldwin, *J. Am. Chem. Soc.*, **66,** 2116 (1944).

An excellent review article dealing with the molecular constitution of starch (and glycogen), and the mechanism of its formation, may be found in the *Quarterly Review of Biology,* **18,** 311 (1943) by Hassid.

The discovery that glycogen as such is present in sweet corn we owe to Morris and Morris, *J. Biol. Chem*, **130,** 535 (1939). See, also, Hassid and McCready, *J. Am. Chem. Soc.*, **63,** 1632 (1941), and Morris, *J Biol. Chem.*, **148,** 699 (1943)

The isolation of glycogen from avian tubercle bacilli is described by Chargaff and Moore, *J. Biol. Chem*, **155,** 493 (1944).

Meyer, *Advances in Enzymology*, **3,** 109 (1943), is the author of an exhaustive article on the chemistry of glycogen.

To appreciate the experimental methods involved, several articles may be consulted:

Moore and Link, *J. Biol. Chem.*, **133,** 293 (1940), offer a new method of characterizing carbohydrates which, they claim, is superior to that of the osazones

Haskins, Hann and Hudson, *J. Am. Chem. Soc*, **64,** 1289, 1490 (1942), follow up an article on cellobiose by announcing a method for synthesizing lactose.

Pascu and Trister, *J. Am. Chem. Soc.*, **61,** 2442 (1939), describe a procedure for the preparation of fully methylated carbohydrates.

Bacon, Baldwin and Bell, *Biochem. J.*, **38,** 198 (1944), describe methods for determining the magnitude of "unit chains" in glycogen.

For enzymic syntheses of starch and glycogen, see references p. 329 under Cori. See, also, Hestrin and Shapiro, *Biochem. J.*, **38,** 2 (1944).

A method of determining the molecular constitution of enzymatically synthesized starch is described by Hassid and McCready, *J. Am. Chem. Soc.*, **63,** 2171 (1941).

Photomicrographs of various sugars are given by Quense and Dehn, *Ind. Eng Chem.* (Analytical Edition), **11,** 555 (1939).

Methods of showing the presence of fatty acids in starches are given by Lehrman, *J. Am. Chem. Soc*, **64,** 2144 (1942); Whistler and Hilbert, *Ibid*, **66,** 1721 (1944); and Schoch, *Ibid.*, **64,** 2954 (1942).

Hanson, Mills and Williams, *Biochem. J.* **38,** 274 (1944), describe the determination of glucuronic acid.

For details regarding the life and work of Emil Fischer, who contributed so much to our knowledge of carbohydrates, see the biography (in German) by Kurt Hoesch (1921), and the delightful autobiography entitled *Aus Meinem Leben* (1922). A much shorter biography will be found in Harrow's *Eminent Chemists of Our Time* (1927).

A historical description of the development of the chemistry of carbohydrates is to be found in Lieben's *Geschichte der physiologischen Chemie* (1935), p. 460.

CHAPTER 3

THE LIPIDS

THE lipids include not only the true fats but substances which are (1) chemically related to fats (like lecithin), or (2) related to them because of common solubilities and possible biological relationships (like cholesterol). The true fats are not only of importance for energy purposes, but a number of the vitamins are often associated with them (Chap. 8), and some of the fats contain unsaturated fatty acids which are needed by the organism (p. 118). The physiological significance of the lipids other than the fats is not always clear; but this matter will be discussed later.

The lipids include

1. Fats: esters of fatty acids and glycerol. (If liquid at ordinary temperature, the fats are known as "oils.")
2. Waxes: esters of fatty acids and certain alcohols (but *not* glycerol).
3. Phospholipids (phosphatides): fats containing, in addition, phosphoric acid and nitrogenous groups (lecithin, cephalin, and sphingomyelin).
4. Cerebrosides (glycolipids): combinations of fatty acid, sugar and a nitrogenous substance (phrenosin, kerasin, etc.).
5. Sterols: hydrogenated phenanthrene derivatives (cholesterol, ergosterol, etc.).

The lipids are soluble, more or less, in ether and allied solvents. The carbohydrates and the proteins (p. 43) are practically insoluble in such solvents.

THE FATS

The fats (and fatty oils like olive oil and cod liver oil) contain mixtures of glycerides: esters of glycerol and one or more fatty acids (often stearic, palmitic or oleic acid). The general formula for such a fat or oil is

$$\begin{array}{l} H_2C\text{—}O\text{—}CO\text{—}R_1 \\ | \\ HC\text{—}O\text{—}CO\text{—}R_2 \\ | \\ H_2C\text{—}O\text{—}CO\text{—}R_3 \end{array}$$

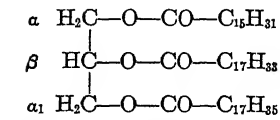
If $R_1 = R_2 = R_3$, then we have a simple glyceride; for example,

$$\begin{array}{l} H_2C\text{—}O\text{—}CO\text{—}C_{17}H_{35} \\ | \\ HC\text{—}O\text{—}CO\text{—}C_{17}H_{35} \\ | \\ H_2C\text{—}O\text{—}CO\text{—}C_{17}H_{35} \end{array}$$

Tristearin.

($C_{17}H_{35}COOH$ = stearic acid)

If the R's are unequal, then we get a mixed glyceride; for example,



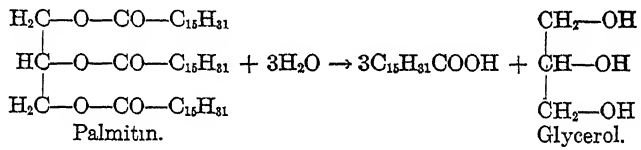
β-Oleo-α-α1-palmitostearin.

($C_{15}H_{31}COOH$ = palmitic acid)
($C_{17}H_{33}COOH$ = oleic acid)

Natural fats are largely mixed glycerides.

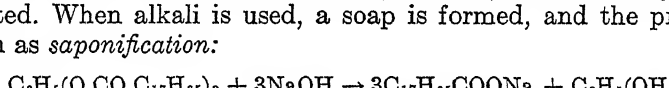
The fats, then, are typical esters.* A simple ester would be formed by the combination of an acid and an alcohol: $CH_3COOH + C_2H_5OH \rightarrow CH_3COOC_2H_5 + H_2O$. A fat would be formed by the combination of an acid (usually of relatively high molecular weight) with the alcohol glycerol.

Being esters, the fats are readily hydrolyzed:



Palmitin. Glycerol.

This hydrolysis can be accomplished by using acid, alkali, superheated steam or the appropriate enzyme (the lipase of the pancreas, for example). When acid (or enzyme) is used, the free fatty acid is liberated. When alkali is used, a soap is formed, and the process is known as *saponification:*



Stearin. Sodium stearate. (A soap) Glycerol.

Fats and oils are, as a rule, more complex than mere mixtures of the triglycerides (stearin, palmitin, olein). For example, the composition of a number of vegetable oils is given in Table 1.

TABLE 1.—COMPOSITIONS OF TYPICAL VEGETABLE OILS

No. of C atoms.	Acid.	Cottonseed oil.		Soybean oil, per cent.	Corn oil, per cent.
		Sea island, per cent.	Upland, per cent.		
14	Myristic	0.3	0.5	.. .	....
16	Palmitic	19.1	20.9	6.5	7.3
18	Stearic	1.9	1.8	4.2	3.3
18	Oleic	33.15	29.2	32.0	43.4
18	Linoleic	39.35	42.8	49.3	39.1
18	Linolenic	....	....	2.2	..
20	Arachidic	0.6	0.1	0.7	0.4
24	Lignoceric	....	....	0.1	0.2

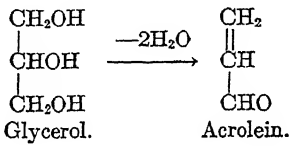
* We owe to Chevreul our knowledge that the fats are esters of fatty acid and glycerol. Chevreul did this work during 1813 to 1823; and Faraday's discovery of benzene, the mother substance of aromatic chemistry, took place in 1825.

The fats we eat—the edible fats—are glycerides of *even-numbered* fatty acids, ranging from butyric (C_4) to lignoceric (C_{24}) and probably higher.

The composition of butter fat is as follows, the numbers representing the per cent of glyceryl ester: butyric acid, 3.; caproic acid, 1.4; caprylic acid, 1.8; capric acid, 1.8; lauric acid, 6.9; myristic acid, 22.6; palmitic acid, 22.6; stearic acid, 11.4; oleic acid, 27.4.*

Simple and mixed glycerides have also been prepared synthetically.

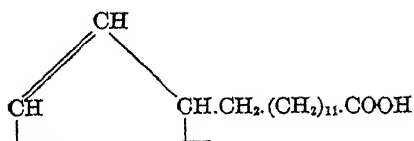
Glycerol, $C_3H_5(OH)_3$, also called glycerin, is obtained in enormous quantities as a by-product in soap manufacture by the process of saponification explained above. It is miscible with water and alcohol. When heated, either alone or in the presence of a dehydrating agent such as $KHSO_4$, acrolein is formed:



Glycerol. Acrolein.

Acrolein has an acrid odor (the odor of burnt fat); and the formation of this substance is used as a test for glycerol (and, indirectly, fat itself).

The Fatty Acids.—Table 2 gives a list of some of the fatty acids found in fats which occur more or less commonly. Those found in nature have almost invariably an even number of carbon atoms. The most important and the most widely distributed are stearic, palmitic, and oleic acids. The highly unsaturated acids, such as linolenic acid, serve (*a*) as essential food constituents (p. 118) or (*b*) contribute to the "drying power" of oils in paint. Chaulmoogric acid, present in chaulmoogra oil (which is expressed from the seeds of certain trees found in Burma and nearby countries), is used in the treatment of leprosy.



Chaulmoogric acid.

Anderson and his associates have isolated a number of fatty acids from the human strain of tubercle bacillus. One of them, tuberculostearic acid, is probably 10-methylstearic acid. Another, phthioic acid, is a saturated fatty liquid with the formula of $C_{26}H_{52}O_2$.

As has already been mentioned, the fatty acids form soaps with metals and esters with alcohols. In the organism they undergo an extensive series of transformations (Chap. 17), their ultimate oxida-

* See page 31 for the formulas of these acids.

TABLE 2.—COMMON FATTY ACIDS. (Reprinted by permission from Bull, *The Biochemistry of the Lipids*, John Wiley & Sons, Inc , Publishers.)

		Occurrence.
I. *Saturated fatty acids.*		
Acetic	CH_3COOH	Vinegar.
Butyric	C_3H_7COOH	Butter.
Caproic	$C_5H_{11}COOH$	Butter, etc.
Caprylic	$C_7H_{15}COOH$	Butter, etc.
Capric	$C_9H_{19}COOH$	Coconut oil, butter, etc.
Lauric	$C_{11}H_{23}COOH$	Spermaceti, coconut oil, etc.
Myristic	$C_{13}H_{27}COOH$	Nutmeg butter, coconut oil, etc.
Palmitic	$C_{15}H_{31}COOH$	Animal and vegetable fats.
Stearic	$C_{17}H_{35}COOH$	Animal and vegetable fats.
Arachidic	$C_{19}H_{39}COOH$	Peanut oil.
Lignoceric	$C_{23}H_{47}COOH$	Arachis oil; cerebrosides.
Carnaubic	$C_{23}H_{47}COOH$	Carnauba wax.
Cerotic	$C_{25}H_{51}COOH$	Beeswax; wool fat; etc.
II. *Unsaturated fatty acids.*		
(*a*) One double bond.		
Oleic	$C_{17}H_{33}COOH$	Animal and vegetable fats.
Erucic	$C_{21}H_{41}COOH$	Rapeseed oil; etc.
(*b*) Two double bonds		
Linoleic	$C_{17}H_{31}COOH$	Linseed oil; cotton seed oil; etc.
(*c*) Three double bonds.		
Linolenic	$C_{17}H_{29}COOH$	Linseed oil.
(*d*) Four double bonds.		
Arachidonic	$C_{19}H_{31}COOH$	Lecithin; cephalin.
III. *Saturated monohydroxy acids.*		
Cerebronic	$C_{24}H_{48}O_3$	Cerebron.
IV. *Unsaturated monohydroxy acids.*		
Ricinoleic	$C_{18}H_{34}O_3$	Castor oil.
V. *Cyclic acids.*		
Chaulmoogric	$C_{18}H_{32}O_2$	Chaulmoogra oil.

tion being largely dependent upon the simultaneous oxidation of carbohydrate.

A number of the unsaturated fatty acids contain 18 carbon atoms. They undergo oxidation when exposed to the air and become brown in color. Mild oxidation (with dilute $KMnO_4$) gives hydroxy acids. For example,

$$\underset{\text{Oleic acid.}}{CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH} + O + H_2O \rightarrow$$

$$CH_3(CH_2)_7CHOHCHOH(CH_2)_7COOH$$
Dihydroxystearic acid.

The method of determining the structure of these acids may be illustrated by the simple example of oleic acid. This acid when hydrogenated is converted to stearic acid. Furthermore, the position of the double bond is determined by forming the ozonide (by the action of ozone) and hydrolyzing the product so formed:

$$R.CH{=}CH.R^1 \xrightarrow{O_3} R.CH\underset{O_3}{<}CH.R^1 \xrightarrow{H_2O} R.CHO + R^1CHO + H_2O_2$$

(The H_2O_2 reacts with one of the aldehydes to give a carboxyl group and water.) The products obtained on hydrolysis are pelargonic aldehyde, $CH_3(CH_2)_7.CHO$, and the half aldehyde of azelaic acid, $CHO-(CH_2)_7.COOH$. The double bond, then, occurs between the ninth and the tenth carbon atoms.

The following list shows the position of the double bonds in other unsaturated fatty acids:

Erucic, $CH_3(CH_2)_7CH{=}CH(CH_2)_{11}COOH$
Linoleic, $CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$
Linolenic, $CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$
Ricinoleic, $CH_3(CH_2)_5CHOHCH_2CH{=}CH(CH_2)_7COOH$

Due to their unsaturated character, the unsaturated fatty acids not only combine with hydrogen but also with halogens. This is the basis for the determination of the *iodine number* (p. 33).

Two types of isomers are possible in the unsaturated acids. One of them depends upon the position of the double bond. Taking oleic acid as the example, 16 isomers are possible; and several have been actually isolated (such as Δ2,3, Δ3,4 and Δ4,5 oleic acids).* Another type of isomerism, geometrical isomerism, depends upon spatial arrangement. Oleic acid (m. p. 13° C.) can be transformed into elaidic acid (m. p. 45° C.) by means of nitrous acid. Elaidic acid has the same empirical formula as oleic acid, and in each case the position of the double bond is 9,10. The difference is a difference in spatial arrangement, one (oleic) being the *cis* form, and the other (elaidic) being the *trans* form:

$CH_3(CH_2)_7CH$
$\|$
$COOH(CH_2)_7CH$
Oleic acid.
(*Cis* form)

$CH_3(CH_2)_7CH$
$\|$
$HC(CH_2)_7COOH$
Elaidic acid.
(*Trans* form)

Ricinoleic acid, the unsaturated hydroxy acid present in castor oil, is optically active, because the carbon attached to the hydroxyl group is asymmetric.

Fat Analysis.—Aside from physical methods (melting point, index of refraction, etc.), the usual analysis of a fat depends upon determining certain chemical constants. Among these are the following:

1. **Saponification Number.**—This represents the number of milligrams of KOH needed to saponify completely 1 gm. of fat (or oil). Roughly speaking, this number varies inversely with the molecular weight of the fat. For example,

	M. W.	*Saponif. No.*
Tributyrin	302.2	557.0
Tricaprin	554 4	303.6
Tripalmitin	806 8	208.6
Tristearin	890.9	188.9
Triolein	884.8	190.2

2. **Reichert-Meissl number** is the number of cubic centimeters of 0.1 *N* alkali required to neutralize the soluble volatile fatty acids from 5 gm. of fat. Butter fat has a particularly high Reichert-Meissl number.

* Δ = double bond.

Volatile fatty acids represent the acids which volatilize on steam distillation. They are confined, approximately, to the series ranging from butyric (C_4) to lauric (C_{12}) and are divided into two groups: those soluble in water and those insoluble in water. Butter fat, coconut and palm oils have a relatively high "volatile fatty acid" content; the reverse is true of most of the fats.

3. **Acetyl number** is the number of milligrams of KOH required to neutralize the acetic acid resulting from the hydrolysis of 1 gm. of the acetylated fat. A fatty acid containing a hydroxyl group will react with acetic anhydride to form the acetylated compound. Castor oil, for example, which contains the unsaturated hydroxy acid, ricinoleic acid, gives a high acetyl number (142-150); the common fats containing smaller quantities of hydroxy fatty acids, yield a considerably smaller number (2.5-20).

4. **Iodine number** is the number of grams of iodine absorbed by 100 gm. of fat. From what has already been said, it is obvious that the iodine number will depend upon the extent of unsaturation in the molecule of fat. To accelerate the absorption process, "halogenating agents" are added; iodine monobromide (*Hanus'* method) or iodine monochloride (*Wijs'* method). A list of some numbers of several fats and oils is given (Table 3):

TABLE 3.—VALUES OF SOME OF THE COMMON FATS (From Winton and Winton, *Analysis of Foods.*)

Fat	*Saponif. No.*	*Iodine No.*
Butter.....	220–241	22– 38
Lard.....	193–203	54– 70
Mutton tallow	192–195	32– 50
Coconut oil..	246–265	8– 10
Cottonseed oil	191–195	104–114
Linseed oil...	190–196	170–202
Olive oil.....	190–195	74– 95
Peanut oil.....	186–189	83–105
Soybean oil.	190–197	115–145

The fats are soluble in ether. This is also true of the sterols (like cholesterol or phytosterol) which are closely associated with fats but, chemically, are not fats at all (p. 38). However, fats are readily saponified, whereas sterols are not. An ether fraction, then, which may consist of a mixture of fat and sterols, can be saponified and the product extracted with ether. Here the fat has been transformed into soap but the sterols remain unchanged; and the latter are now extracted by ether. This ether fraction represents the *unsaponifiable matter*. In such oils as cod liver oil, it is this unsaponifiable matter which contains not only sterols but the vitamins A and D.

Hydrogenation.—As has already been described, unsaturated fatty acids can be saturated by the addition of hydrogen. This process can be accelerated by using a catalyst, such as nickel. By this means, not only unsaturated fatty acids, but unsaturated fats can be made to add hydrogen. The method has its practical application, in that oils, such as cottonseed oil, can be "hardened" or solidified into edible products. Lard substitutes on the market are products of this process.

Slightly less than one-half of the fats and oils produced in this country are used as food. Of the remaining 50-odd per cent which finds its way into various industries (soap, paint and varnish, oilcloth, printing ink, and the like), a little more than half is used for making soap.

Rancidity.—The unpleasant (rancid) odor and taste of fats (including butter) which they acquire on standing are attributed to chemical changes due, largely, to the presence of unsaturated fatty acids.

The change involves the oxygen of the air (which attacks the double bond) and is catalyzed by moisture, heat and light. Peroxides are probably first formed, which are then broken down to aldehydes. Ketones are also formed from the action of microorganisms.

To prevent fat spoilage, the substitution of oxygen by an inert gas, or the removal of oxygen by creating a vacuum, is possible. The more practical method is the use of "anti-oxidants,"substances which prevent oxidation to a greater or less degree. Various such substances have been suggested; a number of them are phenolic in character. Gum guaiac and tocopherol (vitamin E) are among a number of compounds approved by the War Food Administration which may be incorporated in meat fats.

"Drying" Oils.—Somewhat associated with the process of rancidity are the changes undergone by certain oils when exposed to the air. These oils, of which linseed and tung oils are the most important, form a solid surface, which is strong and waterproof, when exposed to the air. The oils are apparently oxidized and polymerized. Such "drying" oils have an iodine number greater than 130 and contain large percentages of highly unsaturated fatty acids. For example, linseed oil contains nearly 50 per cent of linoleic acid and more than 30 per cent of linolenic acid.

The Separation of Fatty Acids.—Steam distillation is used to separate volatile from nonvolatile fatty acids. Unsaturated fatty acids may be separated from the saturated ones by making use of the fact that the lead salts of the former are more soluble in alcohol than those of the latter. The individual fatty acids are often separated from one another by distilling their methyl esters *in vacuo*.

Identification Tests.—This has been discussed in large measure, under fat analysis (p. 32), saponification (p. 29) and the acrolein test (p. 30). The formation of a soap, and the fact that this soap can be salted out of solution with salt and precipitated with the salts of metals (such as $CaCl_2$) is an excellent qualitative test. It should also be noted that the soap first formed is soluble in water, and that such soap can be transformed into the insoluble fatty acids by treatment with HCl.

WAXES

The waxes are esters of fatty acids with alcohols (not glycerol) of high molecular weight. A number of common waxes are given in Table 4.

Industrially, they have their uses in the manufacture of lubricants

(sperm oil), polishes (carnauba wax), ointments ("lanolin" which contains wool wax), candles (spermaceti), etc. In the body these waxes occur as cholesterol esters, in which the fatty acid is joined to the alcohol cholesterol. Anderson has shown that the tubercle bacillus contains a complex wax.

TABLE 4.—A NUMBER OF COMMON WAXES

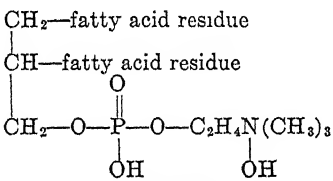
Wax.	*Iodine number.*
Sperm oil	81–90
Carnauba wax	13
Wool-wax	30–35
Beeswax	8
Spermaceti	0–4
Chinese wax	0–1.4

Aside from cholesterol, the common alcohols found in waxes are cetyl alcohol, $C_{16}H_{33}OH$, ceryl alcohol, $C_{26}H_{53}OH$, and myricyl alcohol, $C_{30}H_{61}OH$.

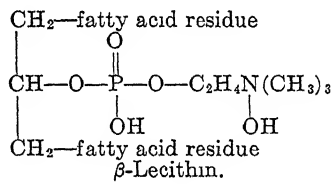
PHOSPHOLIPIDS

The phospholipids are constituents of all animal and vegetable cells. They are present in abundance in brain, heart, kidney, eggs, soy beans, etc. The phospholipids have been separated into three distinct substances: lecithin, cephalin, and sphingomyelin. All three contain the elements nitrogen and phosphorus, beside carbon, hydrogen, and oxygen. In lecithin and cephalin, the N:P ratio is 1:1, and in sphingomyelin, the N.P ratio is 2:1.

Lecithin.—Two types of lecithin are known: the asymmetrical (or α-) form and the symmetrical (or β-) form:

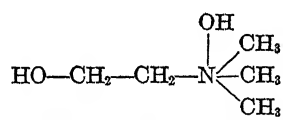


α-Lecithin.*



β-Lecithin.

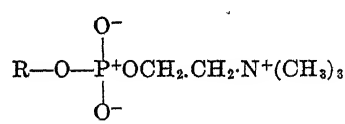
which means that the constituents of lecithin are fatty acids, glycerol, phosphoric acid, and the base, **choline**:



Choline (ethanoltrimethylammonium hydroxide).

The common fatty acids in lecithin are stearic, oleic, and palmitic.

* It is probably more correct to write the lecithins in the zwitterion form (p. 66):

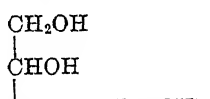


but for the sake of simplicity we adhere to the formulas above.

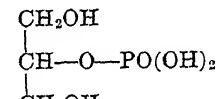
Lecithin is a common cell constituent and is considered to play a part in the metabolism of fat (p. 331). It may be obtained from brain tissue or egg yolk. Crude lecithin from egg yolk is readily prepared by extracting the yolk with ether and precipitating out the lecithin with acetone. Fat and cholesterol, which are also present, remain in solution in acetone, but cephalin (p. 37) is also precipitated; so that the precipitate really consists of a crude mixture of lecithin and cephalin. To separate these two is not an easy problem. One method depends upon the fact that lecithin is more soluble in cold alcohol. The lecithin is finally purified by taking advantage of the fact that it forms a double salt with cadmium chloride, which double salt can be decomposed subsequently with ammonia.

When freshly obtained, lecithin has a waxlike appearance; but it readily turns brown on exposure, due to oxidation. Lecithin is soluble in ether, alcohol, petroleum ether, benzene, carbon tetrachloride, carbon disulfide and chloroform, and is insoluble in methyl acetate and acetone; but the presence of impurities has modifying influences. It can, of course, be saponified, and yields acrolein (p. 30) on heating; but unlike true fats, it contains the elements nitrogen and phosphorus *

The hydrolysis of lecithin results in the production of choline, fatty acids and **glycerophosphoric acid,** of which two varieties are possible:



α-Glycerophosphoric acid.

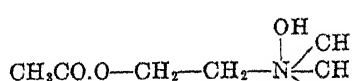


β-Glycerophosphoric acid.

The glycerophosphoric acid is not easily hydrolyzed. Bases have no action at all. Dilute acids do hydrolyze it slowly after boiling for a long time. It can, however, be readily hydrolyzed by an enzyme present in yeast and in animal material. The cobra venom contains an enzyme which splits off one molecule of fatty acid from lecithin (and cephalin, see below), giving a product called lysolecithin which is able to hemolyze red blood cells.

Enzymes capable of hydrolyzing lecithin—there are several varieties—are called *lecithinases.*

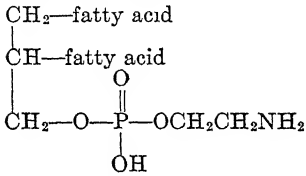
Choline is a base comparable in strength to sodium hydroxide. It is important in preventing the accumulation of fat in the liver (p. 335) and is generally regarded as a constituent of the vitamin B complex (p. 171). Its acetyl derivative, **acetylcholine,** is the substance which is released at parasympathetic nerve endings when they are stimulated (p. 537).



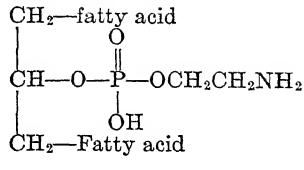
Acetylcholine.

* In the industries, many thousands of pounds of lecithin, obtained from soybean, are used as an emulsifier and in the manufacture of candies, chocolate, cocoa, margarine, medicines, and even in the dyeing of textiles.

Cephalin, or **kephalin,** also consists of two varieties



α-Cephalin.

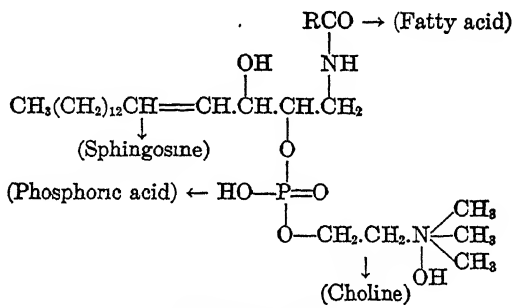


β-Cephalin.

which means that cephalin differs from lecithin in the kind of nitrogenous compound which it contains. This compound in cephalin is β-aminoethyl alcohol or **colamine.*** Cephalin is believed to be concerned with the process of blood clotting (p. 267).

$HO.CH_2\ CH_2.NH_2$
β-Aminoethyl alcohol (ethanolamine or colamine).

Sphingomyelin yields, on hydrolysis, fatty acids, phosphoric acid, choline and another nitrogenous base, sphingosine, with the probable formula $CH_3.(CH_2)_{12}.CH{=}CH.CHOH.CHOH.CH_2NH_2$; which means that it is an 18-carbon compound containing one unsaturated bond, one amino group and two hydroxyl groups. No glycerol is found in sphingomyelin. A probable formula for the compound is:



Sphingomyelin.

The solubilities of the phospholipids may be summarized as follows (S = soluble, X = insoluble and H = soluble in hot solvent):

Phospholipid	*Ether*	*Alcohol*	*Acetone*
Lecithin	S	S	X
Cephalin	S	X	X
Sphingomyelin	X	H	X

These differences in solubility are used for the separation of the phospholipids, although clear-cut separations are rarely possible in this way.

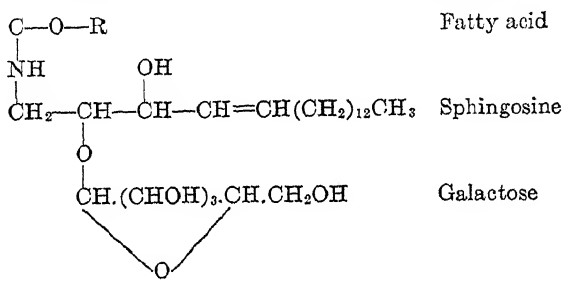
Sphingomyelin, like lecithin and cephalin, forms addition compounds with cadmium chloride. The cadmium salts of lecithin and sphingomyelin are insoluble in ether, whereas the corresponding cephalin salt is soluble in this solvent.

* Folch and Schneider (see reference at the end of the chapter) find that from 40 to 70 per cent of the nitrogen in the cephalin as ordinarily prepared is not due to cholamine at all, but to an amino acid, probably serine (p. 51).

CEREBROSIDES

These substances, sometimes called cerebro-galactosides, or galactolipids, are found more particularly in the brain. Practically nothing is known as to their physiological function. They are readily distinguished from the phosphatides proper by the absence of the phosphoric acid group. They are composed of combinations of fatty acid, sphingosine (p. 37) and the sugar galactose,* and differ from one another only in the kind of fatty acid present. Two of the fairly well known cerebrosides are phrenosin (cerebron) and kerasin. Recently two others, nervone and oxynervone, have been added. **Phrenosin** is believed to yield a characteristic hydroxy acid, cerebronic acid, to which the formula $CH_3(CH_2)_7CH_2CH_2(CH_2)_{12}CHOHCOOH$ has been assigned. **Kerasin** contains lignoceric acid, $CH_3(CH_2)_7CH_2CH_2(CH_2)_{12}CH_2COOH$. The acid in *nervone* is believed to be nervonic acid, $CH_3(CH_2)_7CH{=}CH(CH_2)_{12}CH_2COOH$, which is an unsaturated lignoceric acid. Oxynervonic acid, $CH_3(CH_2)_7CH{=}CH(CH_2)_{12}CHOHCOOH$, an unsaturated hydroxy lignoceric acid, is the acid which characterizes *oxynervone*.

A possible structure for a cerebroside is as follows:



A cerebroside.

These cerebrosides are soluble in hot alcohol, pyridine, and benzene and practically insoluble in ether (hot or cold). The preparation of these substances from brain tissue makes use of such solvent properties, though the actual procedure is cumbersome and still far from satisfactory. They are readily hydrolyzed with acid (on warming) into sphingosine, galactose, and the fatty acid.

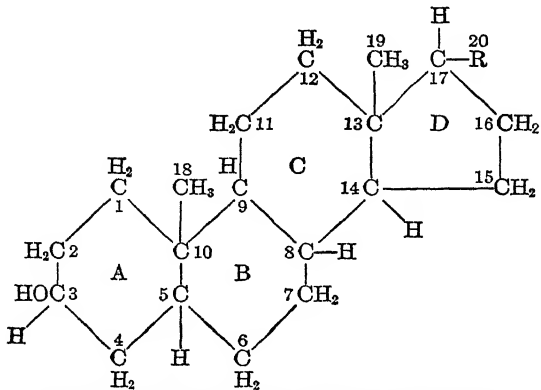
STEROLS

The sterols are complex monohydroxy alcohols found in the plant and animal kingdoms.

They are widely distributed in plant and animal tissues, either in the free state or in the form of esters (a combination with higher fatty acids). Chemically, they are known to be phenanthrene derivatives; or, more correctly, cyclopentanoperhydrophenanthrene derivatives (p. 39); which means that these sterols are, chemically, closely allied to the bile acids (p. 228), the sex hormones (Chap. 24), etc. Through cholesterol and ergosterol a relationship to vitamin D can also be

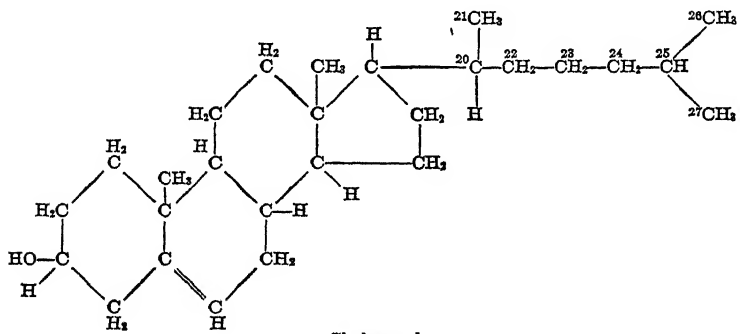
* A cerobroside containing glucose instead of galactose has been reported.

established (Chap. 8) The best known of these sterols is cholesterol. (The general name of **steroid** has been suggested for the group of related compounds: sterols, bile acids, sex hormones, saponins, etc.)



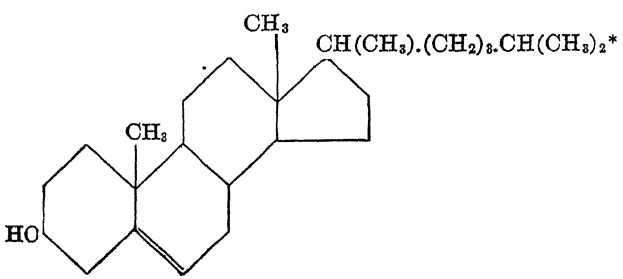
A cyclopentanoperhydrophenanthrene derivative.

Cholesterol, present in all animal cells and particularly abundant in nervous tissue, has the structure



Cholesterol.

or more simply written



which means that it has a hydroxyl group in position 3 and a double bond connecting positions 5 and 6. The saturated hydrocarbon corresponding to cholesterol is known as *cholestane.*

* Evidence for the structure of cholesterol, and some details of its configuration are given in the appendix (p. 549).

In the cholesterol nucleus there are 8 centers of asymmetry, and, theoretically, something like 240 isomers are possible. Fortunately for the problem, but two carbon centers seem to be involved, those at position 3 and position 5.

Cholesterol can be prepared from brain tissue, or, even better, from gallstones. In either case, the essential point in the method is to extract with ether and to allow the cholesterol to crystallize. Unlike the fats or the phospholipids, cholesterol and other sterols cannot be saponified. They represent part of the "unsaponified fraction." (Hickman, using the molecular still, has been able to separate sterols from the natural oils in which they occur by distillation at 100–220° C.)

Cholesterol gives a number of characteristic color tests. One of these is the *Liebermann-Burchard* test, in which a chloroform solution of the sterol is treated with acetic anhydride and concentrated sulfuric acid. The bluish green to green color obtained varies in intensity with the amount of cholesterol present; and this color test is therefore the basis of a quantitative estimation. Another common test is the one developed by *Salkowski*, which consists of mixing the sterol with chloroform and concentrated sulfuric acid, to give a bluish-red to purple color. These tests are not confined to cholesterol, but are given by a number of the sterols. However, in animal tissues one finds comparatively small quantities of sterols other than cholesterol. Saturated sterols (like dihydrocholesterol and coprosterol) fail to give these color tests.

Another test of importance is precipitation with *digitonin*, $C_{56}H_{92}O_2$, (a glycoside belonging to the "saponin" group and occurring in digitalis leaves and seeds). Cholesterol is readily precipitated with digitonin, forming cholesterol digitonide. This is not only a qualitative test, but it can be made the basis for a quantitative determination by weighing the digitonide. The combination with digitonin is possible only if the hydroxyl group in position 3 remains free. Cholesteryl acetate, for example, does not give the test. Certain vegetable sterols, such as stigmasterol, sitosterol, and ergosterol, are also precipitated with digitonin; and it is known that their configuration corresponds to dihydrocholesterol and not to epidihydrocholesterol (p. 550).

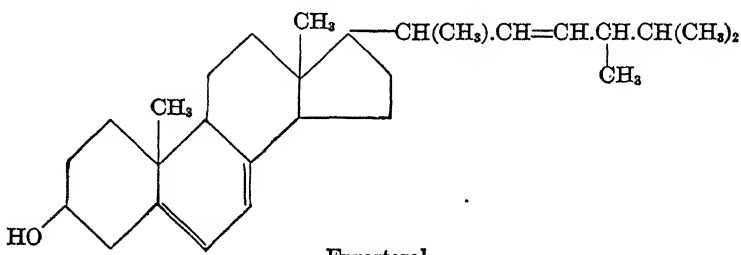
As has been said, cholesterol is the most important sterol found in the animal body. There is abundant evidence to show that cholesterol can be synthesized in the animal organism; and it is equally clear that plant sterols are not utilized by the body. As to the function of cholesterol, little that is definite can be said. The fact that it is always present in cells, and present in such abundance in nervous tissue, suggests its importance. Its chemical relationship to bile acids, sex hormones and a number of synthetic cancer-producing substances, has led to hypotheses which are attractive enough, but far from positive proof. (See, however, pp. 228, 513.)

Wintersteiner has found that aerating cholesterol for some time with molecular oxygen at 37° C. produces 7-ketocholesterol (p. 185) and, in lesser quantity, 7-hydroxycholesterol (see p. 185), etc. It is possible that "this reaction may be one of the pathways by which

cholesterol is degraded in the organism or is converted to vitamin D_3" (see p. 185).

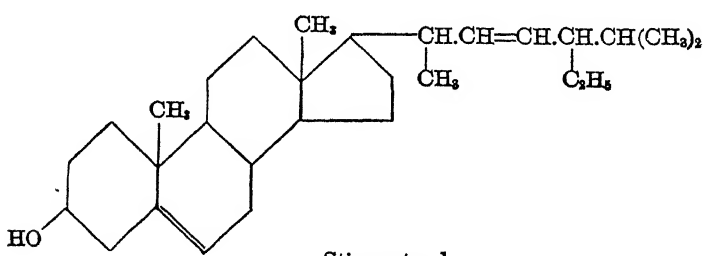
While the specific precursors from which the cholesterol is synthesized in the animal organism are unknown, Bloch and Rittenberg have shown that feeding acetic acid (in the form of deuterium-containing sodium acetate) to mice and rats made it possible to isolate cholesterol from the animal carcass which contained a deuterium concentration over 3 times as high as that of the body fluids. Acetic acid may, therefore, be one precursor in the biological formation of cholesterol.

Ergosterol, originally isolated from ergot and now more readily obtained from yeast, is of especial interest because when irradiated it gives rise to a compound with antirachitic activity—a vitamin D (Chap. 8). Ergosterol has three double bonds in its molecule, and its formula is



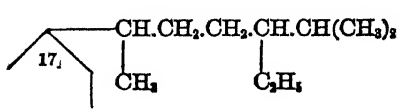
Ergosterol.

Stigmasterol, obtained most readily from soy bean oil, has the formula



Stigmasterol.

The most widely distributed sterol of the higher plants is **sitosterol,** which, in structure, differs from cholesterol by having a different side chain attached to carbon atom 17:



References

A standard work of references is by Bloor, *Biochemistry of the Fatty acids, etc.*, (1943).

Hilditch, an eminent authority, is the author of *The Chemical Constitution of Natural Fats* (1940).

A monograph dealing with some of the material in this chapter is by Bull, *The Biochemistry of the Lipids* (1937). A later article by the same author is to be found in *Symp. Quant. Biol.*, **8**, 63 (1940).

An exhaustive article on choline, by Best and Lucas, will be found in *Vitamins and Hormones*, **1**, 1 (1943).

In the *Ann. Rev. Biochem* for 1944 two articles are recommended. one on lipoids by Brown (p. 93); and the other on steroids by Koch (p. 263). See, also, the same volume for 1945: p. 113, lipids, by Longenecker and Daubert.

The sterols are discussed in masterly fashion by Fieser in his *Chemistry of Natural Products Related to Phenanthrene*, p. 111 (1936).

Fieser and Fieser are the authors of a comprehensive organic text—*Organic Chemistry* (1944), in which they include five chapters on fats and waxes (p. 381) and steroids (p. 925).

Rosenheim and King, the authors of the now generally accepted sterol structures, discuss the subject in the *Ann Rev. Biochem* , **3**, 87 (1934).

Another review of steroid chemistry (sterols, bile acids, sex hormones, etc) is by Strain in Gilman's *Organic Chemistry*, vol. 2, p. 1341 (1943).

Various aspects of lipid chemistry are discussed in the following:

Methods of purifying linoleic acid are given by Brown and Stoner, *J Am Chem. Soc.* **59**, 3 (1937).

Heyningen, Rittenberg and Schoenheimer, *J. Biol. Chem* , **125**, 495 (1938), describe the preparation of fatty acids containing deuterium.

The hydrogenation of vegetable oils for the purpose of preparing edible products is discussed by Wurster, *Ind. Eng. Chem.*, **32**, 1193 (1940).

A review of the hardening and the drying of oils we owe to Fordyce, *Synthetic Organic Chemicals* (Eastman Kodak Co.), **11**, No. 1 (1938)

An improved method of purifying lecithin is given by Pangborn, *J. Biol. Chem.*, **137**, 545 (1941).

Two articles on soy bean lecithin are by Struve, *Science*, **93**, 86 (1941); and by Thornton and Kraybill, *Ind Eng. Chem* , **34**, 625 (1942).

Gray, *J. Biol. Chem.*, **136**, 167 (1940), deals with the structure of cephalin. Folch and Schneider, *J. Biol. Chem.*, **137**, 51 (1941), show that serine is a constituent of ox brain cephalin. See, also, Chargaff and Ziff, *J. Biol. Chem.*, **140**, 927 (1941).

One possible significance of cephalin is discussed by Christensen and Hastings, *J. Biol. Chem.*, **136**, 387 (1940).

The determination of cerebrosides is described by Brand and Sperry, *J. Biol. Chem.*, **141**, 545 (1941).

A method of separating the sterols from the natural oils (by using the molecular still) is given by Hickman, *Ind. Eng. Chem.*, **32**, 1451 (1940).

The occurrence of sphingomyelin in the tissues of a cat are described by Hunter, *J. Biol. Chem.*, **144**, 439 (1942).

Wintersteiner's suggestive experiments on the autoxidation of cholesterol (done in collaboration with Bergstrom) are described in the *J. Biol. Chem.*, **137**, 785 (1941); **141**, 597 (1941); and **143**, 503 (1942).

The separation of sterols from sponges is described by Mazur, *J. Am. Chem. Soc* , **63**, 2442 (1941).

Anderson's work on the lipids of the tubercle bacilli may be found in the *Harvey Lectures*, 1939–40, p. 271. See, also, the *Sigma Xi Quarterly*, March, 1939, p. 39.

Bloch and Rittenberg's suggestive work on the synthesis of cholesterol from acetic acid is given in the *J. Biol. Chem.*, **143**, 297 (1942).

For practical methods of analyzing fats, etc., see Winton and Winton, *Analysis of Foods* (1945), p. 481.

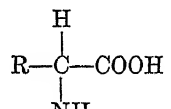
Fats in relation to commercial possibilities are described by Ralston, *Chemical and Engineering News*, **21**, 3 (1943); and by Vahlteich, *Ibid*, **21**, 1238 (1943).

CHAPTER 4

PROTEINS[1]

THE proteins belong to a group of the most complex of chemical substances. They are essential constituents of all protoplasm, and they are also essential food constituents.

The proteins are characterized by the fact that on hydrolysis they yield from 20 to 25 different α-amino acids. (Proline and hydroxyproline, hydrolytic products of proteins, are exceptions in that they are *imino* rather than *amino* acids.)



An α-amino acid

The differences between proteins are largely a matter of the number, the kind and the arrangement of such amino acids within the protein molecule. Since the protein molecule is often built up of hundreds, and even thousands, of these amino acids, the problem of protein structure is one of almost insuperable difficulty. We know, for example, that egg albumin, pepsin, and insulin are typical proteins, in that all three yield a variety of α-amino acids when hydrolyzed; but one of them (pepsin) is also a typical enzyme, and another (insulin) is a typical hormone. What is there in a protein molecule which endows it with enzymic or hormonic properties? We have no real answer as yet.

Stanley has isolated a crystalline protein from the mosaic-diseased Turkish tobacco plants. This protein apparently has the properties of the virus to which the disease has been attributed (see p. 80). Why does this protein show the properties of the virus? We do not know. The antibodies (p. 279) are proteins. Perhaps, even the specific properties of the gene may be due, in part at least, to the proteins present.

While we are on this discussion, a word should be said about "species specificity." The classification of proteins given below might indicate that an albumin is something very definite, irrespective of its source—in the same sense that sodium chloride is a very definite entity, irrespective of its source. This is not so. The albumin obtained from human blood is not the same as that obtained from beef blood. Nor, for that matter, is the casein obtained from cow's milk identical with that obtained from human milk. These differences apply to all proteins. While, chemically, it is well-nigh impossible to show these differences, yet immunological tests are not wanting to show that such differences do exist.

[1] For an elementary introduction to some physico-chemical facts, see appendix, p. 552.

The complexity of the problem makes a satisfactory classification of proteins impossible at present. However, based to a large extent upon more or less obvious differences in physical properties, the following crude classification has been adopted:

I. SIMPLE PROTEINS (NATIVE PROTEINS)

These are native proteins which on hydrolysis are said to yield α-amino acids or their derivatives.

(*a*) **Albumins** are characterized by being soluble in water and being coagulated on heating. Examples are egg albumin,* serum albumin, lactalbumin (from milk), leucosin (from wheat).

(*b*) **Globulins** are insoluble in water, coagulated by heating, soluble in dilute salt solutions and precipitated when the salt concentration is increased. NaCl, $MgSO_4$ and $(NH_4)_2SO_4$ are salts often used. Examples are myosinogen (from muscle), edestin (from hemp seed), ovoglobulin (from egg yolk), serum globulin, amandin (from almonds), legumin (from peas), excelsin (from Brazil nuts).

(*c*) **Glutelins** are insoluble in neutral solvents but soluble in dilute acids and alkalis. Examples are glutenin (from wheat), oryzenin (from rice).

(*d*) **Alcohol-soluble proteins (prolamins** or **gliadins)** are soluble in 70 to 80 per cent alcohol and insoluble in water and in absolute alcohol. Examples are gliadin (from wheat), hordein (from barley), zein (from corn).

(*e*) **Albuminoids (scleroproteins)** are insoluble in neutral solvents. Examples are elastin (from ligament), collagen (from hide, bone, and cartilage), keratin (from horn).

(*f*) **Histones** are soluble in water and insoluble in dilute ammonia. Solutions of other proteins precipitate histones. The coagulum formed on heating is soluble in dilute acids. Examples are globin (from hemoglobin), thymus histone, scombrone (from mackerel).

(*g*) **Protamines** are simpler in structure than the proteins so far considered. They are soluble in water, not coagulated by heating, precipitate other proteins from their aqueous solutions (for example, insulin-protamine, p. 494), possess strong basic properties and form stable salts with strong mineral acids. The few amino acids which are obtained from the protamines on hydrolysis are largely basic in character. Examples are salmine (from salmon), sturine (from sturgeon), clupeine (from herring), scombrine (from mackerel), cyprinine (from carp).†

II. CONJUGATED PROTEINS

These are substances made up of proteins combined with some compound or compounds.

* As showing how even the so-called "simple" proteins may not be "simple" after all, Neuberger presents very strong evidence for the presence, in crystalline egg albumin, of a polysaccharide made up of a number of mannose and glucosamine groups.

† Stedman finds that the nuclei of animal cells contain, among other things, a basic protein which may be either of the protamine or histone type.

(*a*) **Nucleoproteins** are combinations of one or more proteins with nucleic acid. Examples are found in products obtained from glandular tissue and from the germ of grain.

(*b*) **Glycoproteins (glucoproteins)*** are combinations of protein with one or more substances containing a carbohydrate group. Examples are mucin (from saliva), osseomucoid (from bone), tendomucoid (from tendon).

(*c*) **Phosphoproteins** are combinations of protein with phosphorus-containing substances other than nucleic acid or lecithin. Examples are casein (from milk) and perhaps vitellin (from egg yolk).

(*d*) **Chromoproteins** are combinations of protein with various pigments. Examples are hemoglobin, the blood pigment, which is an iron pyrrole complex joined on to protein (p. 258); ferritin, an iron protein compound found in the liver and spleen; catalase, peroxidase and cytachrome C, iron-protein enzymes (p. 395) which play their part in biological oxidations; hemocyanin, a protein containing copper and found in lower invertebrates; laccase and tyrosinase, also enzymes containing copper, which are important in biological oxidations; chlorophyll, the green pigment of plants, which has magnesium as a characteristic element and is combined with proteins in plant tissues.

(*e*) **Lipoproteins** are combinations of proteins with lipids. They occur in cell nuclei, blood, egg yolk, milk, etc. These complexes, rather ill-defined, are believed to be present in the thromboplastic factor (p. 267), some viruses (p. 80) and bacterial antigens (p. 279).

Several other enzymes found in the body, while not chromoproteins, may be regarded as conjugated proteins. For example, the yellow enzyme, a derivative of the vitamin, riboflavin (p. 153) and carboxylase, a derivative of the vitamin, thiamine (p. 148).

III. DERIVED PROTEINS

These substances are divided into (1) *primary protein derivatives* (proteans, metaproteins, and coagulated proteins) and (2) *secondary protein derivatives* (proteoses, peptones, and peptides). (1) represent a comparatively slight hydrolytic change in the protein molecule; (2) represent a more extensive hydrolysis of the protein.

(*a*) **Proteans** are insoluble products resulting probably from the action (for a comparatively short time) of water, dilute acids or enzymes. Examples are myosan (from myosin), edestan (from edestin).

(*b*) **Metaproteins** (infraproteins) are products of the further action of acids and alkalis which are soluble in dilute acids and alkalis but insoluble in solutions of neutral salts. Examples are acid metaprotein or acid albuminate and alkali metaprotein or alkali albuminate.

(*c*) **Coagulated proteins** are insoluble products resulting either from the action of heat or of alcohol.

* The work of Karl Meyer suggests certain modifications. According to him the true glycoproteins contain hexosamine bound to protein or polypeptide, in addition to other sugars; and the substances included are ovomucoid-α (formerly called *ovomucoid*), ovomucoid-β (formerly called *ovomucin*), serum mucoid, the globulin fractions of egg white and serum, thyroglobulin (from the thyroid) and the hormone of pregnancy urine. The "mucins" and "mucoids" according to Meyer, are polysaccharides of various kinds.

(*d*) **Proteoses** are soluble in water and cannot be coagulated on heating. They can be precipitated by saturating their solutions with ammonium sulfate or zinc sulfate.

(*e*) **Peptones** are also soluble in water and are not coagulated on heating, but they are not precipitated by saturating their solutions with ammonium sulfate. Certain alkaloidal reagents—phosphotungstic acid, for example—do precipitate them.

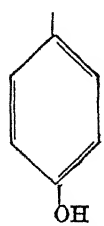
(*f*) **Peptides** are combinations of two or more amino acids, the carboxyl group of one amino acid being joined on to the amino group of another (p. 62).

COLOR REACTIONS OF PROTEINS

The fact that proteins yield α-amino acids on hydrolysis characterizes them quite well; but to carry out such an operation, and to identify the products, is a time-consuming operation. For a preliminary survey, the protein color tests are extremely useful.

Biuret Reaction.—This consists in mixing the protein with sodium hydroxide solution and a very weak solution of copper sulfate. A violet color is obtained. The test probably depends upon the presence of two or more of the following groups (peptide linkages) in the protein molecule: $-CONH_2$ with another $-CONH_2$, or one of the following groups in place of $-CONH_2$: $-CSNH_2$, $-C(NH)NH_2$, $-CH_2NH_2$, $-CRHNH_2$, $-CHOHCH_2NH_2$, $-CHNH_2CH_2OH$, $-CHNH_2CHOH$. Such simple compounds as oxamide, $\begin{matrix}CONH_2\\|\\CONH_2\end{matrix}$, and biuret, $CONH_2.NH.CONH_2$ (from which the test takes its name), give positive biuret reactions.

Millon's Reaction.—When a protein is heated with Millon's reagent (a solution of mercuric nitrite and mercuric nitrate in a mixture of nitric and nitrous acids), a red color or precipitate is obtained. The test is due to the presence in the protein molecule of a phenolic group. The specific compound present in proteins with this structure is tyrosine (p. 56).



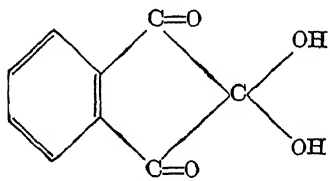
Phenolic group.

Hopkins-Cole (Glyoxylic Acid) Reaction.—A violet ring is obtained when concentrated sulfuric acid is poured into a mixture containing the protein and glyoxylic acid, CHO.COOH. The tryptophan molecule (p. 57) containing the indole nucleus, present in proteins, is responsible for this test. It is believed that tryptophan condenses with

the aldehyde to form the colored product. Gelatin gives a negative test. An actual hydrolysis of gelatin fails to yield any appreciable quantity of tryptophan among its hydrolytic products.

Xanthoproteic Reaction.—A yellow color is obtained when nitric acid and a protein are heated. The yellow color is changed to orange on the addition of alkali. The benzene ring is held responsible for the test. Compounds containing the benzene ring give yellow products on nitration. Among the amino acids, tyrosine (p. 56) and tryptophan (p. 57) give the test.

Ninhydrin (Triketohydrindene Hydrate) Reaction.—The protein boiled with the ninhydrin gives a blue color.



Ninhydrin.

This reaction is due to α-amino acid groups in the protein molecule. Individual α-amino acids give a positive test.

SOME ADDITIONAL PROTEIN REACTIONS

Precipitation Reactions.—Proteins are precipitated by the salts of heavy metals (such as copper sulfate, lead acetate, mercuric nitrate, etc.). It is believed that, in many instances at least, these precipitates are metal proteinates (such as lead proteinate), formed on the alkaline side of the isoelectric point of the protein (p. 65). Proteins are also precipitated by alkaloidal reagents (such as picric acid, phosphotungstic acid, tannic acid, etc.). This precipitation, in many cases at least, represents combinations on the acid side of the isoelectric point of the protein. Neutral salts (such as ammonium sulfate, sodium sulfate, and sodium chloride) are also used to precipitate or "salt out" proteins. Here, some believe, precipitation may be due to dehydration of molecular aggregates in solution. This may also explain precipitation with a dehydrating agent such as alcohol. However, on standing with alcohol proteins undergo other changes (see next paragraph) which affect their solubility.

Coagulation and Denaturation of Proteins.—The change which egg-white undergoes when an egg is heated is but an illustration of the kind of change undergone by a number of proteins when similarly treated. This change, spoken of as *coagulation*, is known to occur in two stages. In the first stage (brought about by heat, acid, alkali, alcohol, urea, ultraviolet light, high pressure, etc.) the protein is so changed that it is no longer as soluble at its isoelectric point as was the original protein, whereas the original protein is soluble. In addition to decreased solubility, the denatured protein may also differ from the native protein by being more readily digested by proteolytic enzymes; by loss of its enzymic properties if the protein is an enzyme; by differ-

ences in antigenic properties; and by an increase in the viscosity of the solution.

This change of *native* protein is called *denaturation*, and the protein which has undergone the change is a *denatured* protein (which, in this form, incidentally, cannot be crystallized).*

The second stage is the precipitation of the insoluble denatured protein, and this probably corresponds to the *coagulation* of colloids in general.

That some change, some rearrangement within the protein molecule, takes place during denaturation is evidenced by the fact that all of the sulfhydryl (—SH) groups found in a protein after hydrolysis can be detected in a denatured protein before hydrolysis, while in the corresponding native protein only a fraction of these groups can be detected.†

Using *x*-ray analysis, Astbury has shown that some denatured proteins are composed of extended peptide chains in parallel bundles; this change in fibrous structure would account for decreased solubility.

Anson and Mirsky have presented evidence to show that the denaturation process, which has been looked upon as an irreversible change, is really a reversible one. Denatured hemoglobin, for example has been reconverted into native hemoglobin which, in turn, is soluble, coagulable and crystallizable. Also, crystalline, soluble, native protein can be obtained from coagulated serum albumin.

Isolation and Composition of Some Proteins.—Proteins are so complex that it is difficult to determine whether the substance isolated is chemically pure. Hemoglobin, egg albumin, etc., can be obtained in the crystalline form. It does not necessarily follow that because they are crystalline they are chemically pure. Largely due to the work of Northrop, Sumner, and Abel, the enzymes pepsin (p. 97), trypsin (p. 48), and urease (p. 96) have been isolated and appear to be proteins; and the hormone insulin has also been isolated, and it, also, appears to be a protein (p. 493). Additions to this group are the isolation by Stanley of the virus of tobacco mosaic, which is described as a crystalline nucleoprotein, and the isolation by Northrop of bacteriophage, presumably a nucleoprotein.

The composition of some of the proteins in terms of their amino acids is given in Table 5 (p. 49). These amino acids are obtained by hydrolyzing proteins by acids, by alkalis or by enzymes (p. 61).

* Denaturation has been more generally defined as "any nonproteolytic modification of the unique structure of a native protein, giving rise to a definite change in chemical, physical or biological properties" (Neurath, Greenstein, Putnam and Erickson).

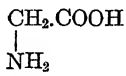
† "Native, unaltered proteins, with very few exceptions, do not contain free titratable sulfhydryl groups. When, however, these proteins are denatured by various agents, such as heat, ultraviolet irradiation, or salts, they give evidence, also with few exceptions, of the presence of free titratable sulfhydryl groups. The reason for the appearance of these and, presumably, a number of other kinds of groups is that the protein molecule unfolds as a result of the denaturation. The sulfhydryl groups, which are probably bound in a nonreactive form within the relatively rigid configuration of the native protein, are released when the configuration of the latter is destroyed as a result of the denaturation, and the groups then become available to added titrating agents." (Greenstein.)

TABLE 5.—THE AMINO ACID CONTENT OF SOME PROTEINS. [Sahyun, *Amino Acids & Proteins.* (Reinhold Publish. Corp.)]

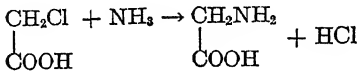
Amino acids (percentage).	Casein.	Edestin.	Egg albumin.	Fibrin (cattle)	Gelatin.	Gliadin.	Hemoglobin.	Keratin (wool).	Pepsin.	Salmin	Serum albumin.	Serum globulin.	Zein.
Alanine	1 9	3 6	2 2		8 7	2 0	4.2	4 4	. .	.	2 7	2 2	9 8
Ammonia	2.3	2 3	1 2	.	0 4	5 2	1 2	1 4	1 4	.	1 3	.	3 6
Arginine	3.8	16.7	5.6	7 7	8 7	2 6	3.6	10 2	1 3	87 4	4 9	5 2	2 0
Aspartic acid	6.0	12 0	8 1	5 9	3 4	1 4	8 9	7 3	6 8	.	3 1	2 5	1 8
Cystine	0.4	1 4	1 8	1.5	0 2	2 4	1 0	13 1	2 2	.	5 7	1 1	0 9
Glutamic acid	21 8	20.7	16.1	14.1	5 8	46 9	6 3	15 0	18 6		1.6	8 2	31 3
Glycine	0 5	3.8	0	. .	25 5	0	.	0 6	..	.	0	3 5	0
Histidine	2 5	2 4	1 5	2.5	2 9	3.3	7.6	0.7	0.3	..	3 4	0 9	0.9
Hydroxyproline	0 2	2 0	. . .	....	14 7	. .	. . .	.. .	. ..				1 0
Isoleucine and Leucine	9 7	21.0	10 7	...	7 1	6 6	29 0	11 5	. .	..	20 0	18 7	25 0
Lysine	6.3	2 4	5.1	10 1	5 9	0 7	8.1	2 8	1 7	.	13 2	6 2	0
Methionine	3.3	2 4	2 4	2 6		2 1		0 7	.	.			2 4
Phenylalanine	3.9	3.1	5 1	.	1 4	2 4	4 2	..			3 1	3 8	7 6
Proline	8 7	4.1	4 2	5 1	17 5	13 2	2 3	4 4	.	11 0	1 0	2.8	9 0
Serine	5.8	0.3	.. .	.	3 3	0 1	1.0	2.9	.	7 8	0 6	.	1 0
Threonine	4.0	....	....	.. .	1.4		.						
Tryptophan	1 2	1.5	1 3	3 0	0	1 1	1 3	1 8	2.2	..	0 5	2 3	
Tyrosine	6.6	4.6	4.2	6.5	0	3 0	3 2	4 8	10 3	....	4.8	6 7	5 9
Valine	7.9	6.3	2.5	....	0	3.4	....	2 8	....	4.3	....	....	1.9

Some details regarding the hydrolysis of proteins and the methods employed in isolating the amino acids will be given presently. In the meantime, we shall list the amino acids

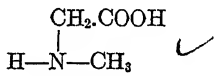
Glycine, glycocoll or aminoacetic acid, is the simplest of the amino



acids. It may be prepared from chloroacetic acid by the action of ammonia:

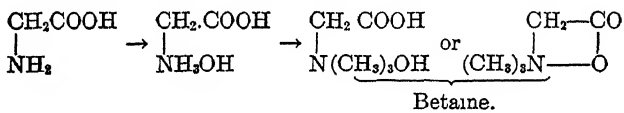


A methyl derivative of glycine, **sarcosine,** is formed when creatine



Sarcosine.

(p. 378) is decomposed. A completely methylated glycine is **betaine.**



Betaine.

Glycine was among the first of the amino acids isolated from the hydrolysis of proteins.

Alanine, α-aminopropionic acid, is optically active. In fact, with

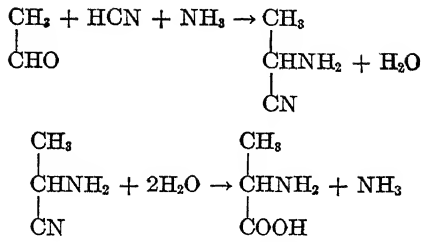
Alanine.

the exception of glycine, all the amino acids derived from proteins show optical activity.*

Alanine may be synthesized from acetaldehyde by the action of hydrogen cyanide and ammonia:

* Natural alanine is *configurationally* related to *l* (+) lactic acid (compare with footnote, p. 12). The naturally occurring amino acids belong to the *l*-series of configurationally related compounds. "It seems highly desirable," writes Dunn [*Ann. Rev. Biochem.*, **10**, 91 (1941)] "that the natural amino acids should be designated as *l* (+) or *l* (−) and the unnatural ones as *d* (+) or *d* (−)." Using this system, naturally occurring alanine becomes *l* (+) alanine, and the naturally occurring serine becomes *l* (−) serine. Formerly these were written—and to some extent still are—as *d*-alanine and *l*-serine, respectively.

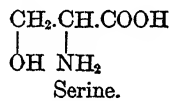
When obtained synthetically, alanine is of the *dl*-variety. Krebs has shown that kidney tissue contains an enzyme (p. 90), called by him "*d*-amino acid oxidase" (p. 353), which oxidizes amino acids of the "unnatural" (*d*) configuration into ammonia and the corresponding α-keto acids. By allowing this oxidase to act on the *dl*-alanine, the *d*-form is destroyed and the *l*-alanine can be recovered.



Alanine is present in practically all proteins. Fibroin (from silk) contains 25 per cent of this acid.

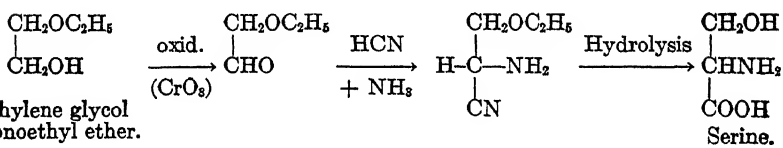
β-alanine, $CH_2(NH_2)CH_2COOH$, a constituent of carnosine and anserine (p. 447) which are found in muscle, is also part of the pantothenic acid molecule, pantothenic acid being a vitamin (p. 166). So far, neither β-alanine nor β-amino acids in general, have been isolated from proteins.*

Serine, β-hydroxy-α-aminopropionic acid,



Serine.

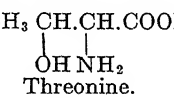
has been prepared, among others, by Dunn:



Ethylene glycol monoethyl ether.

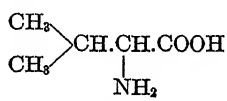
Serine.

Threonine, β-hydroxy-α-aminobutyric acid, has been isolated by Rose from protein hydrolytic products and is considered by him to be an essential amino acid in nutrition (p. 114).



Threonine.

Valine, α-aminoisovaleric acid, has the structure



Valine.

* β—alanine has been obtained synthetically in the following way, among others: First, ammonia is added to acrylonitrile:

$$CH_2{=}CHCN + NH_3 \rightarrow NH_2CH_2CH_2CN + NH(CH_2CH_2CN)_2$$

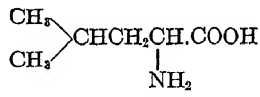
The primary amine, β-aminopropionitrile, is hydrolyzed to give the hydrochloride of β-alanine

$$NH_2CH_2CH_2CN + 2HCl + 2H_2O \rightarrow NH_2CH_2CH_2COOH\,HCl + NH_4Cl$$

Liberation of β-alanine from its hydrochloride can be accomplished by using an anion—exchange resin (see reference at end of Chapter).

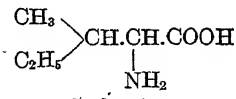
It is found to the extent of about 8 per cent in casein and is an essential amino acid.

Leucine, α-aminoisocaproic acid, is very widely distributed. It is an essential amino acid.



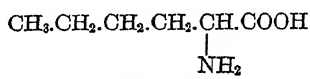
Leucine.

Isoleucine, α-amino-β-methyl-β-ethylpropionic acid, like leucine, is considered an essential amino acid.



Isoleucine.

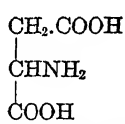
Norleucine, α-aminocaproic acid, is found in a number of proteins, but only in traces.



Norleucine.

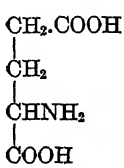
So far we have listed monoaminomonocarboxylic acids. Now we shall list several monoaminodicarboxylic acids.

Aspartic acid, α-aminosuccinic acid, is widely distributed.



Aspartic acid.

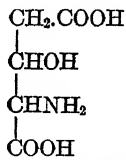
Glutamic acid, α-aminoglutaric acid, is also widely distributed, and is present in particularly large quantities in casein and gliadin (from wheat).*



Glutamic acid.

* It has been pointed out (p. 50, footnote), that the naturally occurring amino acids belong to the *l*-series of configurationally related compounds. This would, therefore, hold true of glutamic acid among the rest. However, Kögl claims that the glutamic acid obtained from the proteins in *cancerous* tissue contains appreciable quantities of the *d*(−)-form. If true, this observation would constitute an important distinction between normal and cancerous tissue. So far, Kögl's observations have been both confirmed and denied. (See p. 371, footnote.)

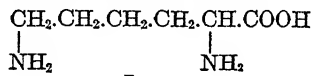
Hydroxyglutamic acid, β-hydroxy-α-aminoglutaric acid, is found in small quantities if at all.*



Hydroxyglutamic acid.

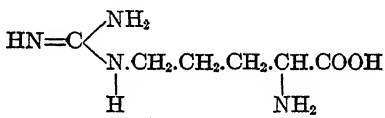
The following are diaminomonocarboxylic acids:

Lysine, α, ε-diaminocaproic acid, is an indispensable amino acid in nutrition. It occurs in most of the proteins.



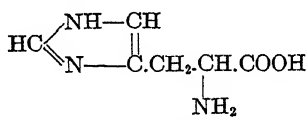
Lysine.

Arginine, δ-guanidyl-α-aminovaleric acid, makes up more than two-thirds of the total amino acids in clupeine (from herring) and salmine (from salmon) and occurs, in smaller amounts, in most proteins. It is a strong base, forming stable carbonates. Within certain limitations, it is an essential amino acid (p. 114).



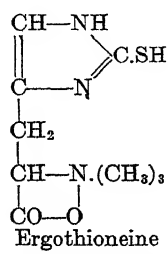
Arginine.

Histidine, β-4-imidazolylalanine, β-4-imidazolyl-α-aminopropionic acid, contains one amino group in the side chain and one imino (—NH—) group in the ring. It is another of the amino acids which be-



Histidine.

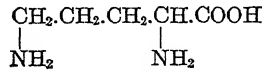
long to the indispensable or essential group. It is present in many proteins. A derivative of histidine, **ergothioneine,** the betaine of thiohistidine, is found in ergot and in blood.



Ergothioneine

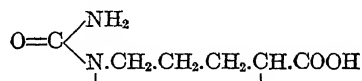
* This acid has given rise to much discussion and a great deal of confusion. The methods of determining it and the methods used for characterizing it are open to question.

Ornithine, α,δ-diaminovaleric acid, is obtained by the hydrolysis of arginine (the action of alkali or the enzyme arginase).



Ornithine.

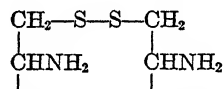
Citrulline, δ-carbamido-α-aminovaleric acid, is considered by many to be of importance in the formation of urea in the body (p. 357). It was first isolated from the juice of the watermelon.



Citrulline.

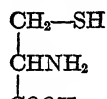
Two amino acids are known to contain sulfur:

Cystine, dicysteine, di-(β-thio-α-aminopropionic acid),



Cystine.

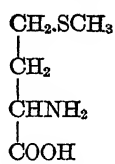
is the naturally occurring amino acid. A reduced form of this acid, *cysteine,* is of importance in biological oxidations.



Cysteine.

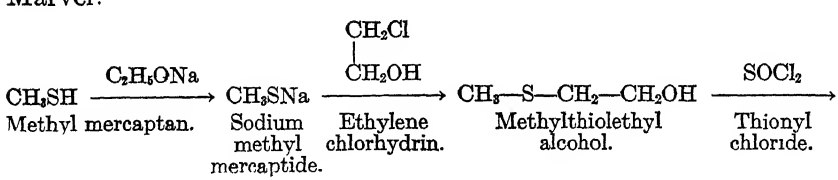
Cystine is obtained in large quantities from material containing keratin (hair, feathers, etc.).

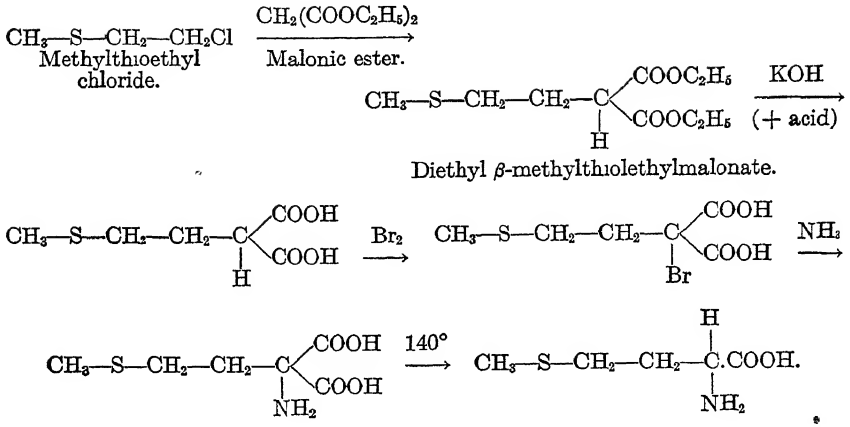
Methionine, γ-methylthiol-α-aminobutyric acid. Mueller first isolated it and Barger and several other chemists have since synthesized



Methionine.

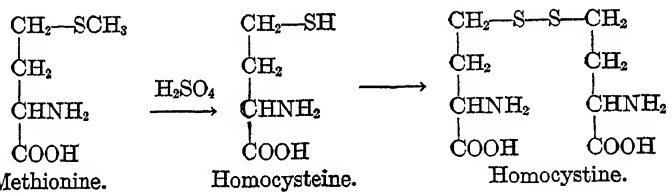
it. The following synthesis, making use of malonic ester, is due to Marvel:



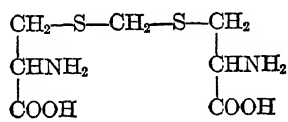


Methionine is an essential amino acid, whereas cystine is not.

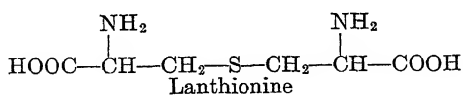
Du Vigneaud has obtained *homocystine*, the next higher homolog of cystine, by heating methionine with sulfuric acid.



Djenkolic acid, the cysteine thioacetal of formaldehyde, has been isolated from the djenkol bean (*Pithecolobium lobatum*). It does not belong to the amino acids found when proteins are hydrolyzed.



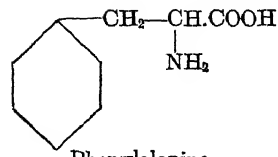
Lanthionine, β-amino-β-carboxyethyl sulfide, is a product of the acid hydrolysis of wool (which, prior to hydrolysis, has been boiled for one hour with 2 per cent sodium carbonate solution). It is not a *direct* product of protein hydrolysis.



Using a similar preliminary treatment with sodium carbonate, lanthionine can be obtained from any source of keratin (such as human hair and chicken feathers) and from lactalbumin.

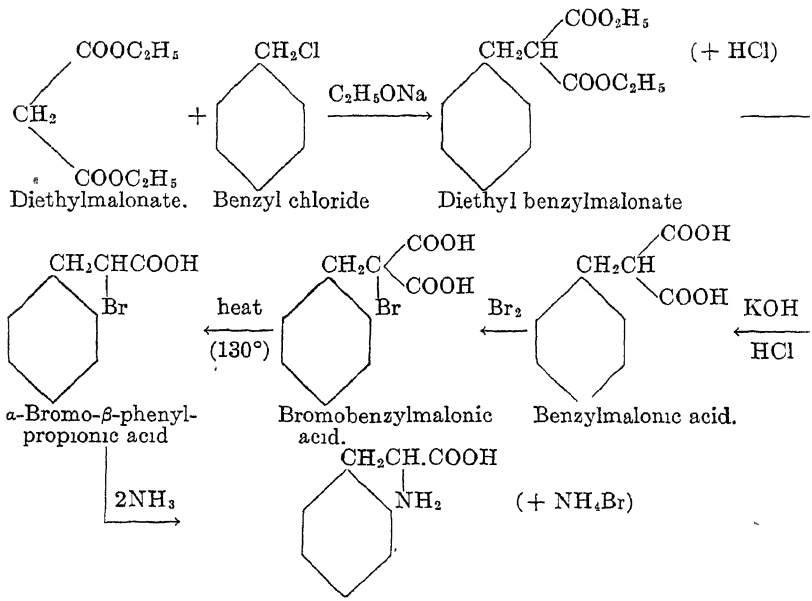
There are several amino acids with aromatic nuclei:

Phenylalanine, β-phenyl-α-aminopropionic acid, is found in many proteins, the protamines excepted. It is, according to Rose, an indispensable amino acid.

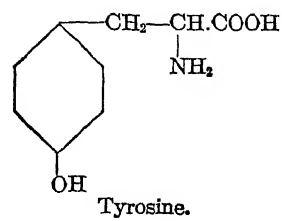


Phenylalanine.

Marvel has synthesized this amino acid as follows:

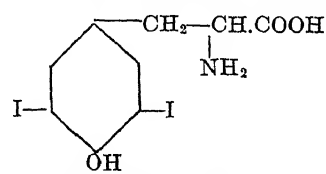


Tyrosine, *p*-hydroxyphenylalanine, *β-p*-hydroxyphenyl-*α*-amino-propionic acid, is a common constituent of proteins (the Millon reaction, p. 46, depends upon its presence), though practically absent in gelatin.



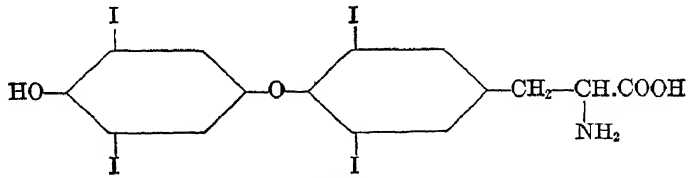
Tyrosine.

Iodogorgoic acid, 3,5-diiodotyrosine, has been obtained from the thyroid gland



Iodogorgoic acid.

Thyroxine, 3,5-diiodo-4(3′,5′-diiodo-4-hydroxyphenoxy)-phenylalanine, is the hormone of the thyroid gland about which more will be said presently (p 479) It is found in the gland combined with protein, the combination being known as thyroglobulin.

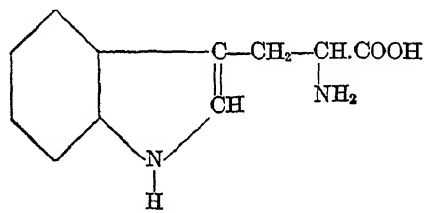


Thyroxine.

Neither iodogorgoic acid nor thyroxine are among the common products of protein hydrolysis.

Several amino acids have heterocyclic nuclei:

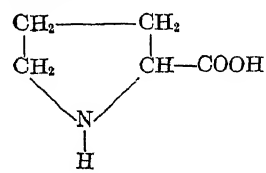
Tryptophan, α-amino-β-3-indolepropionic acid, is an essential amino acid The Hopkins-Cole test (p. 46) is dependent upon its presence. Nearly all proteins contain this amino acid in variable



Tryptophan.

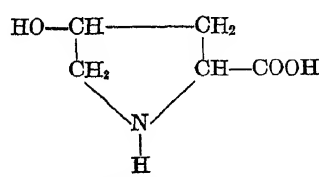
amounts. Zein, a protein obtained from corn, contains very little tryptophan, and gelatin contains none of it.

Proline,* pyrrolidine-α-carboxylic acid, is readily soluble in alcohol. It is found in most proteins.



Proline.

Hydroxyproline,* γ-hydroxypyrrolidine-α-carboxylic acid, is present in small quantities in proteins.



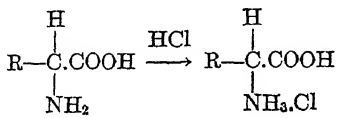
Hydroxyproline.

* Notice that proline and hydroxyproline contain the imino rather than the amino group.

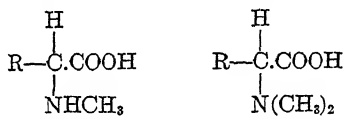
Synthesis of Amino Acids.—The simplest procedure is that illustrated in the preparation of glycine (p. 50): the action of ammonia on the halogenated acid. The principle has been used in the synthesis of a number of the amino acids (leucine, valine, aspartic acid, etc.). Another method involves the cyanohydrin synthesis, illustrated in the case of alanine (p. 50): the action of ammonia and hydrogen cyanide on the appropriate aldehyde. Still a third method of synthesis involves malonic ester, illustrated in the preparations of methionine (p. 54) and phenylalanine (p. 56). This does not exhaust the list.

Some Chemical Reactions of Amino Acids.—These reactions apply to the proteins also since they contain free amino and carboxyl groups.

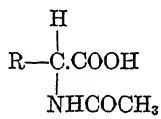
1. They form salts with acids, such as hydrochlorides:



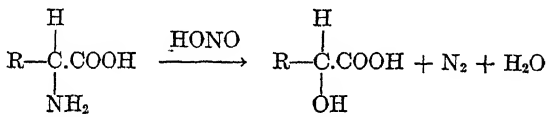
2. Methylation produces such compounds as



3. Acetyl chloride or acetic anhydride gives

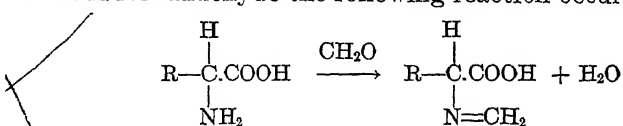


4. Nitrous acid decomposes the amino group liberating nitrogen.



This is the principle employed by Van Slyke in determining the rate of protein hydrolysis. If we assume, for the time being, linkages such as —CO—NH— in the protein molecule, then, as hydrolysis of a protein proceeds, more and more amino groups are set free; which means, in turn, a corresponding evolution of nitrogen when the products are treated with nitrous acid. Proline and hydroxyproline do not react with nitrous acid.

5. With formaldehyde the following reaction occurs:*



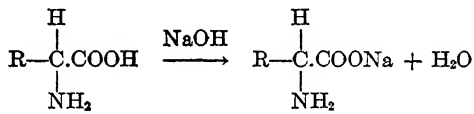
* Using the "zwitterion" form (p. 66), we may write the equation

$$^{-}COO.R.NH_3^{+} + CH_2O \rightarrow {}^{-}COO.RNH_2.CH_2O + H^{+}$$

Here the basic character of the amino group is destroyed by the replacement of the hydrogen by a methylene group, giving the carboxyl group an opportunity to assert itself freely. From the discussion above it can be seen that hydrolysis of a protein sets free not only amino but carboxyl groups; and these are increased in amount as the hydrolysis proceeds. The treatment with formaldehyde destroys the effect of the amino group, and the extent of the formation of free carboxyl groups can be determined by titration with standard alkali.* This is the principle of Sörensen's *formol titration.*

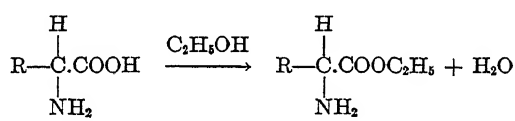
6. The amino acids often form characteristic crystalline combinations with Reinecke's salt, $[Cr(CNS)_4(NH_3)_2]NH_4$. Proline, histidine, and arginine, among others, form such combinations. This reaction is made use of in the isolation of amino acids. These metal complexes have been studied by Bergmann. He finds that a metal complex of the type $[Cr(C_2O_4)_3]K_3$ is a specific precipitant for glycine.

7. Due to the presence of the carboxyl group, reactions with bases take place:



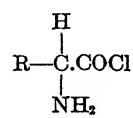
8. The amino acids form copper salts which are also of use in isolating the amino acids.

9. With alcohol and hydrochloric acid, esters are formed:

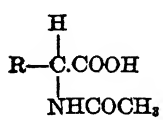


Fischer's original method of separating a number of the amino acids was to distil fractionally their esters (*in vacuo*).

10. The acyl halides of the type

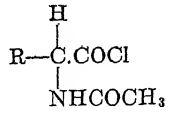


can be formed provided the amino group is first "protected" by being, for example, acetylated:



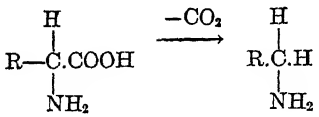
*See, however, p. 66.

Then treatment with thionyl chloride, $SOCl_2$, or phosphorus pentachloride gives

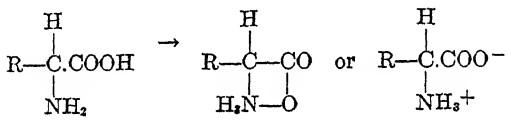


The "protecting" group ($—COCH_3$) can later be removed with HCl.

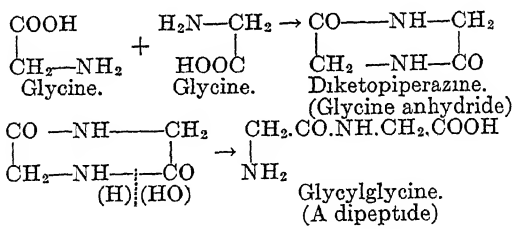
11. Heating with barium hydroxide removes CO_2 and gives the primary amines. In putrefactive processes, this change is brought about by bacteria



12 The amino acids form inner salts or dipolar ions (**zwitterions**):

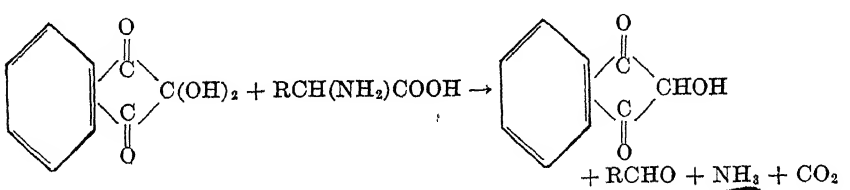


13. The amino acids undergo dehydration to give diketopiperazines, which yield peptides (in this case the dipeptides) on boiling with hydrochloric acid:



This was a method used by Fischer in his attempts to synthesize polypeptides from amino acids.

14. The reaction of proteins with ninhydrin has already been mentioned (p. 47). Van Slyke finds that when α-amino acids are boiled in water with an excess of ninhydrin at pH 1 to 5, the CO_2 of their carboxyl groups is quantitatively evolved in a few minutes and can be determined gasometrically



The Hydrolysis of Proteins and the Separation of the Amino Acids. Hydrolysis of a protein is brought about by boiling with acid, or by allowing a proteolytic enzyme such as trypsin to act on the protein. Alkali is practically never used (except in the Folin method of estimating tryptophan), since it racemizes the amino acids and destroys arginine and cystine. Sulfuric acid is often favored as the hydrolytic agent because, after the necessary boiling (from fifteen to twenty hours), the excess acid is conveniently removed with barium hydroxide. Hydrochloric acid is found more useful when the ultimate goal is the isolation of the monoamino acids.

The separation and the isolation of the various amino acids in the mixture after hydrolysis involves a complicated chemical procedure and is still far from satisfactory. Only the briefest outline can be given here.

Usually, an attempt is made first to separate the amino acids into groups, such as the monoamino acids, the basic (two amino groups) amino acids and the dicarboxylic (two carboxyl groups) acids. Butyl alcohol will extract many of the monoamino acids. The dicarboxylic acids can be separated as their calcium salts, insoluble in alcohol (the calcium salts of the other amino acids are soluble). The basic amino acids are precipitated with phosphotungstic acid. Sometimes the separation into groups is accomplished by electrodialysis. Here the basic amino acids tend to migrate to the cathode, the dicarboxylic acids tend to proceed to the anode. The separation of individual amino acids, particularly in quantitative amounts, is a matter of great difficulty. Some of the monoamino acids can be separated by the fractional distillation of their methyl esters. Tyrosine and cystine may often be obtained directly from a protein hydrolysate, due to their comparative insolubility. Tryptophan may be precipitated as the mercury salt, histidine as a silver salt, arginine as a flavianate, glycine as a double salt with potassium trioxalatochromiate.*

* The difficulty of quantitatively separating the various amino acids in a protein hydrolysate makes the task of determining such amino acids very unsatisfactory. Rittenberg and Foster have adopted an isotope dilution method which promises much better results. The principle involved is that a compound which has an abnormal isotope content is inseparable by the usual laboratory procedures from its normal analogue. If, for example, the isotope-containing glycine is added to a hydrolytic mixture of normal amino acids, and then glycine is isolated, this will be a representative sample of the mixture of the added isotope-containing glycine and the glycine originally present.

From the amount of glycine added (x) and its content of N^{15} (C_0), as well as the N^{15} content of the isolated glycine (C), the amount (y) of glycine originally present in the mixture can be calculated from the equation

$$y = \left(\frac{C_0}{C} - 1\right) x$$

Microbiological methods are also coming into use. In principle, by leaving out one of the amino acids essential for the growth, say of *Lactobacillus arabinosus*, a medium for the determination of that particular amino acid is prepared.

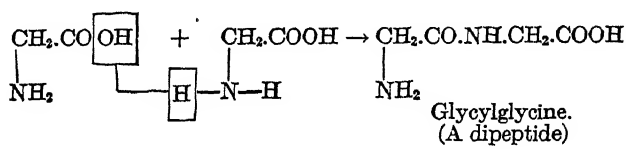
Still another method—the solubility product method—for determining amino acids in a hydrolysate with greater accuracy has been suggested by Bergmann and Stein (see references at the end of the Chapter).

Analysis of a Protein by Distribution of Nitrogen.—A method originally developed by Van Slyke is often used. First the amide nitrogen is estimated by boiling the hydrolyzed solution with slaked lime and estimating the ammonia evolved. The excess of slaked lime precipitates "humin," a black insoluble material, and the nitrogen in this "humin" is determined. Phosphotungstic acid precipitates the basic amino acids and cystine. This precipitate is dissolved in alkali, and from the amount of sulfur in this solution the amount of cystine can be calculated. The arginine can be estimated by boiling it with sodium hydroxide and determining the ammonia evolved. A determination of amino nitrogen (with nitrous acid) in another sample makes it possible to assign values to the other basic acids. In the filtrate from the phosphotungstic precipitate determinations are made for total and amino nitrogen. (See Table 6.)

TABLE 6.—DISTRIBUTION OF NITROGEN IN PROTEINS (per cent of total nitrogen).

	Amide	*Humin*	*Cystine*	*Arginine*	*Histidine*	*Lysine*	*Mono-amino*	*Mono-non-amino*	*Total*
Casein	10 27	1 28	0 2	7 41	6 21	10.3	55 81	7.13	98. 81
Egg yolk	9 0	2 4	...	14 5	3 1	9 4	60 6	1.6	100.6
Egg white	9 2	2 0	.	11 7	0 2	10 1	66 0	1 6	100.8
Hemoglobin	5 24	3 6	0	7.7	12 7	10.9	57 0	2 9	100.04
Gelatin	2 25	0 07	0	14 7	4 48	6 32	55 8	14.9	98.95
Lactalbumin	7 72	1 65	2.34	7 27	4.2	13 08	60 91	2 03	99 2
Edestin	9.99	1 98	1 49	27.05	5 75	3 86	47 55	1 7	99 37
Gliadin	25.52	0 86	1 25	5 71	5.2	0.75	51 98	8 5	99.77

The Synthesis of Polypeptides.—When two amino acids are coupled together a dipeptide is formed. The coupling of three such amino

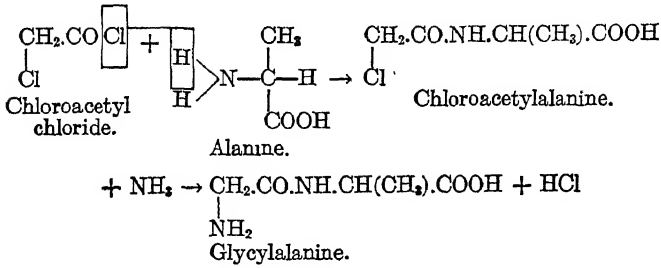


Glycylglycine.
(A dipeptide)

acids gives a tripeptide; etc. In general, these combinations of amino acids are known as polypeptides. It is believed that linkages found in polypeptide chains are similar to those found in protein molecules; and this makes the synthesis of such polypeptides a matter of importance. Several methods of synthesis are available:

1. The dehydration of the amino acid to form a piperazine and the hydrolysis of this product. This method, which we owe to Fischer, has already been given (p. 60). The method has severe limitations. It is almost impossible to get a peptide containing different amino acids.

2. This method can be illustrated by the following example:



$$+ NH_3 \rightarrow \underset{\underset{NH_2}{|}}{CH_2.CO.NH.CH(CH_3).COOH} + HCl$$

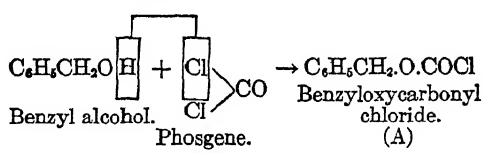
Glycylalanine.

If in the place of chloroacetyl chloride we use α-bromopropionyl chloride we provide the alanyl radical, and the resulting product would be alanylalanine. In other words, by using the appropriate halogen derivative, different dipeptides can be obtained. Obviously, the alanine itself can be replaced by some other amino acid. The method can be used to prepare tripeptides and polypeptides in general, within limitations.

$$\underset{\underset{NH_2}{|}}{CH(C_4H_9).CO.NH.CH_2CO.NH.CH(CH_3).COOH}$$

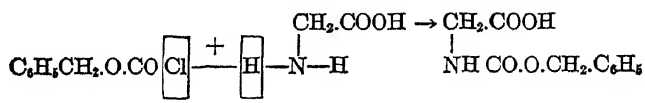
Leucylglycylalanine.
(A tripeptide)

3. By far the most practical method is one developed by Bergmann. The key to the method is the selection of the group used to "block" the amino group. The one selected is known as the "carbobenzoxy" group. Benzyl alcohol may be made to combine with phosgene to form the carbobenzoxy derivative:

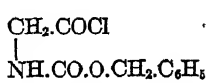


(A) is the reagent selected to "block" the amino group.

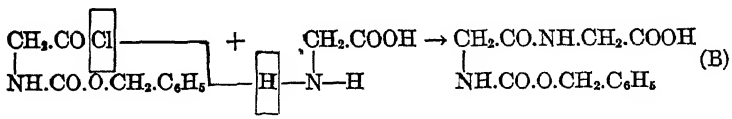
(A) combines with an amino acid thus:



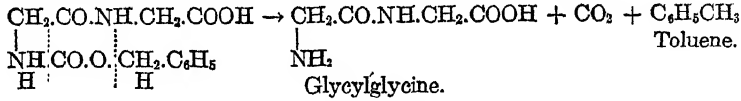
and this can be transformed into the corresponding acid chloride with phosphorus pentachloride:



the resulting compound can now be combined with an amino acid:

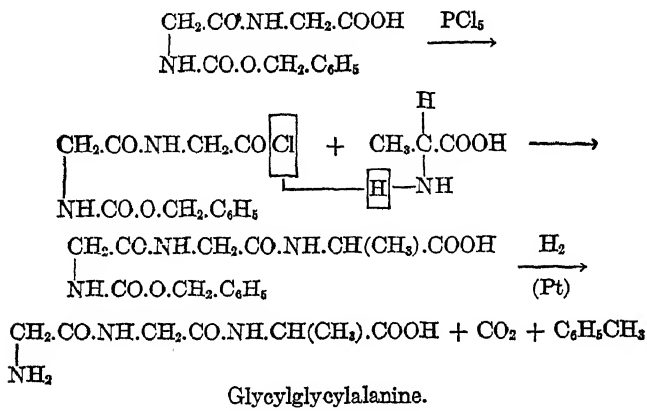


The carbobenzoxy group can be eliminated by treatment with hydrogen (in the presence of palladium black). The free peptide is formed by the removal of toluene and carbon dioxide.



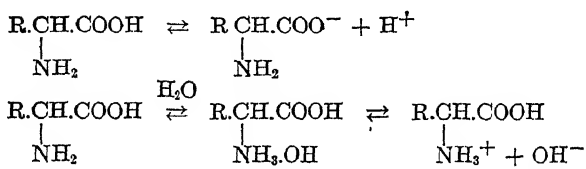
Here no hydrolytic agent is needed to split off the carbobenzoxy group —a procedure which also tends to split the peptide linkage itself.

If a tripeptide is required, (B) is halogenated and coupled with another amino acid:

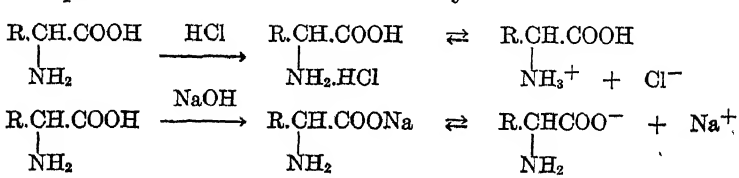


This method has the added advantage in that it can be applied to optically active amino acids, since the carbobenzoxy amino acids, in contrast to other acyl derivatives of amino acids [as illustrated in methods (2) and (3)], are not racemized.*

Proteins Are Amphoteric Electrolytes (or Ampholytes).—Proteins are amphoteric electrolytes due to the presence of amino and carboxyl groups:



and in the presence of acids and alkalis they react as follows:



* For an application of the method to the synthesis of glutathione, see appendix (p. 563).

It may be interesting to note here that nylon, the synthetic textile, resembles a polypeptide in structure. For its preparation, see appendix (p. 564).

A protein, then, is a substance of the type HPrOH Its ionization as an acid is

$$HPrOH \rightleftarrows H^+ + PrOH^- \quad (1)$$

and its ionization as a base is

$$HPrOH \rightleftarrows OH^- + HPr^+ \quad (2)$$

Applying the law of mass action to (1) we find that

$$K_a = \frac{(H^+)\ (PrOH^-)}{(HPrOH)} \quad (3)$$

and from (2) we get

$$K_b = \frac{(OH^-)\ (HPr^+)}{(HPrOH)} \quad (4)$$

K_a (the dissociation constant for the acid) and K_b (that for the base) are rarely equal. Such a protein dissolved in water will not show equal concentrations of H^+ and OH^-.

Hardy showed that in an electrical field the protein will migrate in one direction or in the other, depending upon whether acid or alkali is added. There must be some condition, however, where the tendency to migrate is at a minimum (or where there is the tendency to migrate equally in both directions). When this is reached we arrive at the **isoelectric** point of the protein.

At the isoelectric point the tendency of proteins to combine with acids or alkalis is at a minimum. On the acid side of the isoelectric point the proteins should combine with acids, and on the alkaline side of this point they should combine with bases. By a simple series of experiments, Loeb showed this theory to be correct.

Loeb's Experiment Illustrating the Isoelectric Point.—Samples of gelatin were kept in contact with nitric acid of different concentrations. After pouring off the acid and washing the gelatin, each sample was added to a solution of silver nitrate. The gelatin was next filtered, washed, melted, brought to a known volume, and an adequate portion was used for a *p*H determination, while another was exposed to sunlight. (The experiments involving the silver nitrate were performed in a dark room.) After a time all solutions with a *p*H higher than 4 7 turned brown or black, while those of *p*H less than 4.7 remained colorless.

Michaelis had found quite independently and by other means (cataphoresis experiments—migrations in an electrical field) that the isoelectric point of gelatin is at a *p*H of 4.7. Loeb assumed that at *p*H greater than 4.7, gelatin combines with silver to form a salt of the type of silver gelatinate (which darkens on exposure); whereas below *p*H 4.7, gelatin combines with acid to form gelatin nitrate which does not react with silver salts; hence there is no change in color on exposure.

Using potassium ferrocyanide in the place of silver nitrate, where now the tendency would be to form combinations with the ferrocyanide ion, such combinations took place only below, but not above *p*H 4.7; again presenting evidence in favor of Loeb's point of view.

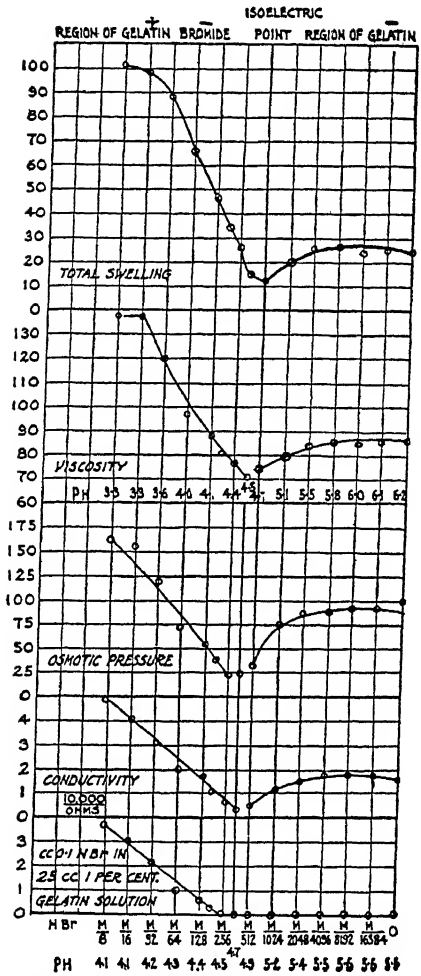


Fig. 1.—Curves showing that the total swelling, viscosity, osmotic pressure, and conductivity of gelatin are minimal at the isoelectric point, *p*H 4 7. (Loeb, *Proteins and the Theory of Colloidal Behavior*, McGraw-Hill Book Co., Publishers.)

The effect of the isoelectric point on a number of properties of protein is given in Fig. 1.

"Zwitterions" or "Dipolar Ions."—It has been assumed so far that when acids and alkalis combine with proteins (or amino acids), the following takes place:

$$Cl^- + {}^+NH_3.R.COOH \xleftarrow{HCl} NH_2.R.COOH \xrightarrow{NaOH} NH_2.R.COO^- + Na^+ \quad (1)$$

which means that the addition of hydrochloric acid causes the ionization of the amino (basic) group as hydrochloride, and the addition of sodium hydroxide causes the ionization of the carboxylic (acid) group as sodium salt. This implies that the protein (or amino acid) in aqueous

solution is practically in an unionized condition. More recent work points to the conclusion that this view is incorrect. The newer view, known as the "zwitterion" or "dipolar ion" theory, claims that the amino acid itself is in a completely dissociated form:

$$^{+}NH_3.R\,COO^{-}$$

and that the reactions of acids and bases are to be formulated thus:

$$Cl^{-} + {}^{+}NH_3.R.COOH \xleftarrow{HCl} {}^{+}NH_3.R.COO^{-} \xrightarrow{NaOH} NH_2.R.COO^{-} + Na^{+} \quad (2)$$

While the results in (1) and (2) are apparently the same, the interpretation is quite different. In (2), the amino group is already ionized. The addition of hydrochloric acid merely serves to depress the ionization of the carboxylic group, which is therefore displaced by the stronger mineral acid. Again, the addition of sodium hydroxide displaces the weaker amino group by the stronger base, leaving the ioniza-

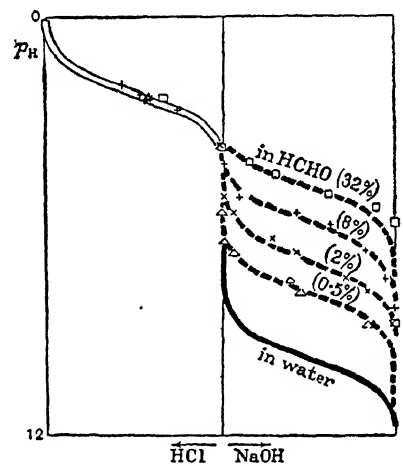


Fig. 2.—Titration curves of glycine in presence of increasing concentrations of HCHO. (Harris, *Biochem. J*, **24**, 1080.)

tion of the carboxyl group unchanged. According to the older view, adding hydrochloric acid measures the basic dissociation constant; according to the newer view, the reverse is true: we are really measuring the acid dissociation constant. According to the older view, adding sodium hydroxide measures the acid dissociation constant; the newer view means that that represents the measure of the basic dissociation constant.

The evidence in favor of the "dipolar ion" hypothesis is by now quite extensive; but only one example of the type of evidence can be given here. As we have already seen (p. 58), proteins (and amino acids) react with formaldehyde thus:

$$\begin{array}{l} R.CH.COOH \\ \quad | \\ \quad NH_2 \end{array} + OCH_2 \rightarrow \begin{array}{l} R.CH.COOH \\ \quad | \\ \quad N{=}CH_2 \end{array}$$

The amino groups alone are affected here; the carboxyl groups remain unchanged. If, now, we titrate an amino acid, such as glycine, with hydrochloric acid, in the absence and in the presence of formaldehyde, any change in the titration curve must be due to the amino group. Figure 2, taken from a paper by Harris, shows changes in the titration curves when sodium hydroxide is titrated with glycine in the presence of varying percentages of formaldehyde. However, when hydrochloric acid is substituted for sodium hydroxide, the curve is the same, with or without formaldehyde.

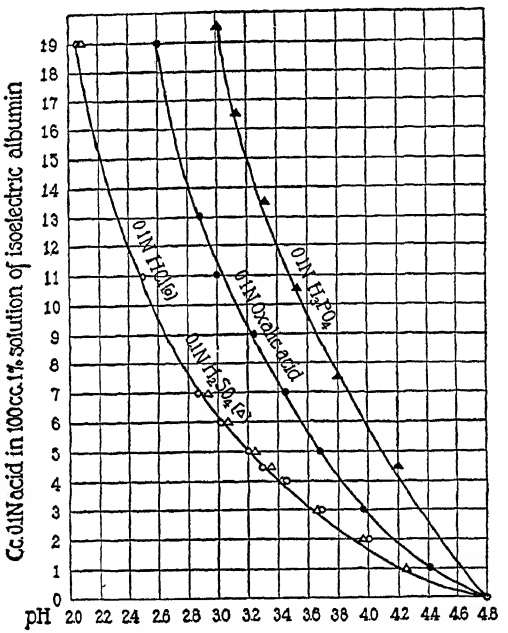


Fig. 3.—The ordinates represent the number of cubic centimeters of 0.1*N* HCl, H_2SO_4, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric crystalline egg albumin to the *p*H indicated on the axis of abscissa. Enough H_2O was added to bring the albumin and acid to a volume of 100 cc. For the same *p*H the ordinates for HCl, H_2SO_4, and phosphoric acid are approximately as 1:1:3. The ratio of HCl to oxalic acid is a little less than 1:2 when *p*H is > 3.0. (Loeb, *Proteins and the Theory of Colloidal Behavior*, McGraw-Hill Book Co., Publishers.)

Proteins Combine with Acids and Bases in Stoichiometrical Relations.—That proteins, which are colloids, nevertheless combine with acids and alkalis in the way crystalline compounds do (in definite chemical combination) was proved by Loeb.

Phosphoric acid gives off one H ion below *p*H 4.6. Oxalic acid acts like a monobasic acid below *p*H 3.0 and like a dibasic acid above this *p*H. Sulfuric acid even at *p*H 3.0 (and below) acts as a dibasic acid. Assuming, then, that the reactions between proteins and acids and alkalis are of a chemical nature, one would require three times as much 0.1*N* H_3PO_4 to bring a given volume of isoelectric gelatin to a *p*H below 4.6 (say *p*H 3.0) as by using nitric or hydrochloric acid Sulfuric and hydrochloric acids should show equality. Under these

conditions twice as much oxalic acid should be needed as hydrochloric acid.

Figure 3 represents the results of experimental data. The curve is the same for 0.1*N* HCl and 0.1*N* H_2SO_4, both being strong acids. In examining the curve for phosphoric acid, it will be noticed that for each *p*H the ordinate for phosphoric acid is approximately three times as high as that for hydrochloric acid (allowing for experimental errors).

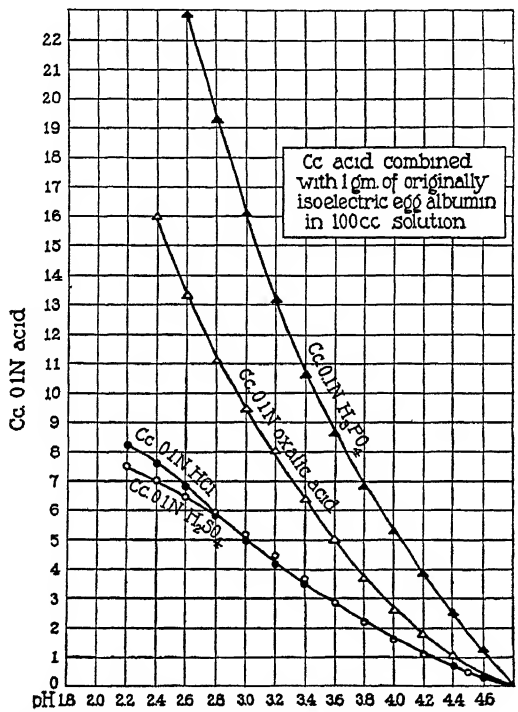


Fig. 4.—Proof of the stoichiometrical character of the combination of acids with isoelectric albumin. The same mass of albumin combines with three times as many cubic centimeters of 0.1*N* H_3PO_4 as of HCl or H_2SO_4, and with twice as many cubic centimeters of 0.1*N* oxalic acid below *p*H 3 0. (Loeb, *Proteins and the Theory of Colloidal Behavior*, McGraw-Hill Book Co , Publishers.)

The values for oxalic acid for *p*H below 3.2 are almost twice as high as those for hydrochloric acid.

From these curves the amount of acid in combination with 1 gm. of isoelectric protein (in this case crystallized egg albumin) in a 1 per cent solution of this protein at different *p*H can be calculated. This gives us Fig. 4. Other proteins (for example, gelatin) give similar results.

If in the place of acids we use bases to combine with proteins, the results lead to similar conclusions (Figs. 5 and 6).

Donnan's Theory of Membrane Equilibria.—Loeb developed his theory of the colloidal behavior of proteins from two principles: one, that proteins are amphoteric electrolytes (this subject has already been discussed); the other, that Donnan's theory of membrane equilibria (which is about to be discussed) furnishes an explanation of the influ-

ence of electrolytes on osmotic pressure and membrane potentials. The colloidal properties of proteins according to Loeb, are associated with the presence of a nondiffusible ion.

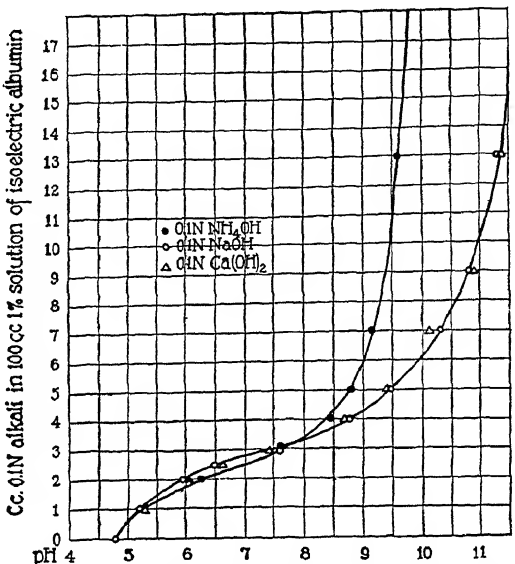


Fig. 5.—Curves representing the number of cubic centimeters of 0.1*N* NH_4OH, NaOH, and $Ca(OH)_2$ required to bring 1 gm. of isoelectric, crystalline egg albumin in 100 cc. of solution to different *p*H. The curves for NaOH and $Ca(OH)_2$ are identical. (Loeb, *Proteins and the Theory of Colloidal Behavior*, McGraw-Hill Book Co., Publishers.)

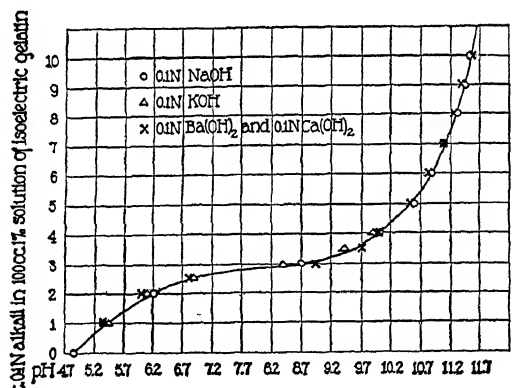


Fig. 6.—Curves for the number of cubic centimeters of 0.1*N* NaOH, KOH, $Ba(OH)_2$, and $Ca(OH)_2$ required to bring the same mass of about 0.8 gm. of isoelectric gelatin in 100 cc. solution to different *p*H. All four curves are identical. (Loeb, *Proteins and the Theory of Colloidal Behavior*, McGraw-Hill Book Co., Publishers.)

Donnan, in developing his theory, assumed a membrance of such a nature as to be impermeable to one of the ions present. Assume an ionized salt, NaR, on one side of the membrane and sodium chloride on the other side. These electrolytes are of such a nature that, with

one exception, they are readily diffusible through the membrane. This exception is represented by R^- (which in NaR may represent the protein part of the molecule). Before equilibrium is reached the situation is as represented in (A). At equilibrium the change is as repre-

$$\begin{array}{l|l} Na^+ & Na^+ \\ R^- & Cl^- \end{array} \qquad \begin{array}{l|l} (Y+Z)\ Na^+ & Na^+\ (X) \\ (Z)\ R^- & Cl^-\ (X) \\ (Y)\ Cl^- & \end{array}$$

(A) (B)

sented in (B). By thermodynamical means, Donnan reached the conclusion that the product of the concentration of the diffusible ions on one side of the membrane is equal to the product of the concentration of the diffusible ions on the other side of the membrane. In other words,

$$X^2 = Y(Y + Z)^*$$

or $X > Y$. In other words, there is an unequal distribution of diffusible ions at equilibrium.

Donnan predicted that a potential difference would exist between two such solutions at equilibrium which could be expressed in the form of Nernst's equation:

$$E = \frac{RT}{F} ln \frac{X}{Y}$$

where E = potential difference, R = gas constant, T = absolute temperature, F = the number of coulombs in a Faraday of electricity, ln = the natural logarithm, and $\frac{X}{Y}$ = the ratio of the concentrations of any diffusible univalent ion on the two sides of the membrane. Loeb verified this theory experimentally. Donnan's theory also predicts differences in osmotic pressure; this prediction also was verified.

The Molecular Weights of Proteins.—The protein molecule is so complex that until recently little was known with regard to its molecular weight. Svedberg has introduced a highly successful method

* Donnan states that when this equilibrium is established the energy required to transport reversibly and isothermally 1 gm. molecule of Na^+ from (2) to (1) equals the energy which can be gained by the corresponding reversible and isothermal transport of 1 gm. molecule of Cl^-. In considering the following infinitely small isothermal and reversible change of the system,

$$\delta n \text{ mol Na } (2) \rightarrow (1)$$
$$\delta n \text{ mol Cl } (2) \rightarrow (1)$$

the energy which can be gained in this way (the diminution of free energy) is zero; hence

$$\delta n RT \log \frac{[Na^+]_2}{[Na^+]_1} + \delta n RT \log \frac{[Cl^-]_2}{[Cl^-]_1} = 0$$

$$\text{or } [Na^+]_2.[Cl^-]_2 = [Na^+]_1.[Cl^-]_1$$

(1) in diagram (B) represents Na^+ in combination with Cl^- and R^-, whereas (2) in the same diagram represents Na^+ joined to Cl alone. Therefore

$$[Na^+]_1 > [Na^+]_2 \text{ and } [Cl^-]_1 < [Cl^-]_2.$$

whereby the mass of heavy molecules is determined by measuring the sedimentation velocities in the ultracentrifuge (see Table 7).*

TABLE 7.—MOLECULAR WEIGHTS OF PROTEINS.

Protein.	*Source.*	*Molecular Weight (approximate).*
Cytochrome C	Beef heart	15,600
Lactalbumin	Cow's milk	17,400
Gliadin	Wheat	27,500
Lactglobulin	Milk	35,200
Pepsin	Gastric juice	35,500
Insulin	Pancreas	41,000
Egg albumin	Hen's egg	44,000
Hemoglobin	Man	63,000
Serum albumin	Horse	70,000
Yellow enzyme	Yeast	82,000
Serum globulin	Human blood	140,000
Catalase	Beef liver	250,000
Urease	Jack bean	480,000
Thyroglobulin	Thyroid	650,000
Bushy stunt virus	Tomato	7,600,000
Mosaic virus	Tobacco	60,000,000

The Structure of Proteins.—Assuming the peptide linkages in proteins, a view based largely on Emil Fischer's pioneer researches, an almost infinite number of proteins is possible by varying the number, the nature and the order of the constituent units. Fortunately, the

TABLE 8.—THE NUMBER OF AMINO ACID RESIDUES IN THE MOLECULES OF CATTLE HEMOGLOBIN, CATTLE FIBRIN, CHICKEN EGG ALBUMIN, AND SILK FIBROIN. (Bergmann and Niemann, *Science*, **86**, 187.)

Amino acid.	Number of amino acid residues per molecule.			
	Cattle hemoglobin.	Cattle fibrin.	Chicken egg albumin.	Silk fibroin.
All amino acids	$2^6 \times 3^2$	$2^6 \times 3^2$	$2^5 \times 3^2$	$2^5 \times 3^4$
Arginine	$2^2 \times 3^1$	$2^5 \times 3^0$	$2^2 \times 3^1$	$2^2 \times 3^1$
Lysine	$2^2 \times 3^2$	$2^4 \times 3^1$	$2^2 \times 3^1$	$2^2 \times 3^0$
Histidine	$2^5 \times 3^0$	$2^2 \times 3^1$	$2^2 \times 3^0$	$2^0 \times 3^0$
Aspartic acid	$2^5 \times 3^0$	$2^5 \times 3^0$	$2^4 \times 3^0$	
Glutamic acid	$2^4 \times 3^0$	$2^3 \times 3^2$	$2^2 \times 3^2$	
Glycine	.....	.... .	. . .	$2^4 \times 3^4$
Alanine	....	. .		$2^3 \times 3^4$
Tyrosine	$2^2 \times 3^1$		$2^3 \times 3^0$	$2^1 \times 3^4$
Proline	$2^2 \times 3^1$	$2^5 \times 3^0$		
Tryptophan	.....	$2^1 \times 3^2$		
Cysteine	$2^0 \times 3^1$	$2^0 \times 3^2$	$2^1 \times 3^0$	
Methionine	.....	$2^2 \times 3^1$	$2^2 \times 3^1$	

work of Bergmann suggests that the situation may not be so hopeless. The proteins, it now seems, conform to a stoichiometrical law which

* "The ultracentrifuge," writes West, "rotates many thousand times per minute and develops centrifugal force thousands of times greater than the pull of gravity. By placing colloidal solutions in an ultracentrifuge and determining the rate at which the particles fall in the solution under known centrifugal force, it is possible to calculate the molecular weights and sizes of the particles."

limits the number of possible formulas. This law is illustrated in Table 8.*

The order in which the amino acids are linked, as developed by Bergmann and his collaborators, is an important contribution to protein structure, but it still does not settle the question of how they are arranged in space. If the proteins are built of peptide chains, these can be represented as follows (Fig. 7):

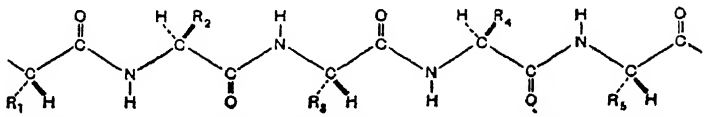


Fig. 7.—Straight peptide chain. The dotted bonds are behind, the heavy bonds in front, and the light bonds in the plane of the paper.

* In cattle hemoglobin the molecule is composed of $2^6 \times 3^2$ (= 576) "all amino acids," and of this number there are $2^2 \times 3^1$ (= 12) arginine residues, $2^5 \times 3^0$ (= 32) histidine residues, $2^2 \times 3^2$ (= 36) lysine residues, etc.

$$F_i = \frac{N_t}{N_i} \quad (1)$$

where F_i = frequencies of the individual amino acids
N_t = total number of amino acid residues
N_i = number of individual amino acid residues.

In turn,
$N_t = 2^n \times 3^m$, where n and m are positive whole numbers
$N_i = 2^{n'} \times 3^{m'}$, where n' and m' are either zero or positive whole numbers
$F_i = 2^{n''} \times 3^{m''}$, where n'' and m'' are either zero or positive whole numbers

$$n = n' + n'' \text{ and } m = m' + m''$$

and $N_t = N_i' + N_i'' + N_i''' + \ldots\ldots\ldots N_i^x$.

Experimentally determined values of N_i', N_i'', N_i''', etc., and of F_i', F_i'', F_i''', etc., give values for N_t that are whole number multiples of 288 ($= 2^5 \times 3^2$). Many proteins contain 288 units, or a whole number multiple thereof. Proteins, then, are constructed on relatively simple structural lines. Each amino acid residue in the peptide chain of the protein molecule probably recurs at constant intervals.

From equation (1) we gather that each glycine residue in silk fibroin is separated from other glycine units by some amino acid grouping other than glycine:

—G—X—G—X—G—X—

and each alanine grouping is separated from other alanine groupings by three other residues:

—A—X—X—X—A—X—X—X—A—

and each tyrosine residue is separated from other tyrosine units by 15 other groupings:

$$—T—(X)_{15}—T—(X)_{15}—T—$$

Combining these three may give us part of the fibroin molecule in this form:

G–A–G–X–G–A–G–X–G–A–G–T–G–A–G–X–G–A–G–X–G–A–G–X–G–A–G–T–G

Common to all proteins is their peptide structure (—CO—NH—CHR—), but they differ in that their individual amino acid constituents show different frequencies (F_i). "The physico-chemical and the biological properties of a particular protein," writes Bergmann, "are based, in the last analysis, on the frequencies with which its constituent amino acid residues recur within its peptide chain."

When the number of amino acid residues present in one molecule of a given protein is multiplied by the average weight of the residues, the minimum molecular weight of the protein is obtained. For example, the molecular weight of egg albumin is according to one method, 35,700 (that is, $123.9 \times 2^5 \times 3^2$), which is within the range given by Svedberg.

Bergmann's theory has been criticized in several quarters. See, for example, Chibnall, *Nature*, **150**, 127 (1942).

where R_1, R_2, R_3 represent the tails of amino acids and the group $-\underset{\underset{NH_2}{|}}{\overset{\overset{H}{|}}{C}}-COOH$ the head.

x-Ray analysis of the protein fibroin suggests that it is composed of straight peptide chains lying parallel. In feather keratin the distance between two adjacent tails, R_1 and R_2, is reduced by slight

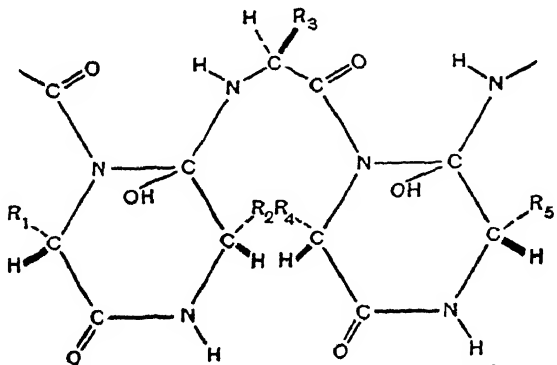


Fig. 8.—Peptide chains of keratin in contracted state.

folding of the chain; but the chain can be stretched until it is almost straight. In wool keratin—the wool can be stretched to double its length without tearing—it is believed that the unstretched form consists of chains coiled up into hexagons (Fig. 8).

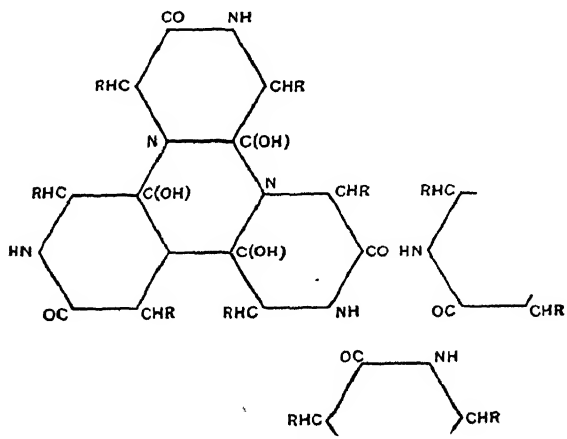


Fig. 9.—A "cyclol 6" molecule.

Muscle fibers when relaxed resemble unstretched wool, though in muscle the fibers tear when stretched. This possibly means that the chains in muscle fibers resemble those of wool, "but that the hexagons so formed cannot be pulled out because they are fixed by some chemical combination" (Marrack; Astbury).

Wrinch suggests that amino acids may also be bent into a series of hexagons, resembling the ring in contracted keratin. Six such amino acid residues may be shown thus (Fig. 9):

However, it should be mentioned that Pauling and others believe that a protein with the cyclol structure would be less stable than one with the polypeptide chain structure.*

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Lewis is the author of a readable article on proteins in nutrition, in the *Handbook of Nutrition* (1943), p. 13. On this phase of the subject, see further Chap. 7 of the present volume.

Excellent articles on the amino acids may be found in Gilman's *Organic Chemistry* (1943), vol. 2, p. 1079 (by Clarke), and in a pamphlet put out by Merck and Co. entitled *The Story of the Amino Acids* (1940).

The advances in protein chemistry are reviewed in the *Ann. Rev. Biochem.* See, for example, Hewitt, and Kerwick and McFarlane, **12,** 81, 93 (1943); Neurath and Greenstein, **13,** 117 (1944); Steinhardt, **14,** 113 (1945).

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In *Science,* **79,** 439 (1934) will be found a general discussion by Bergmann of his method of synthesizing polypeptides.

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Protein structure is considered by Bergmann, *Science,* **86,** 187 (1937); *J. Biol. Chem*, **112,** 577 (1938); Niemann, *Symp. Quant Biol.*, **6,** 58 (1938); Astbury, *Ann. Rev. Biochem.*, **8,** 113 (1939); *Adv. Enzym*, **3,** 63 (1943); Wrinch and Langmuir, *J. Am. Chem. Soc*, **60,** 2247 (1938); *Symp Quant. Biol*, **9,** 218 (1941), Pauling and Niemann, *J. Am. Chem. Soc.*, **61,** 1860 (1939), Cohn, Fankuchen, Oncley, Vickery and Warren, *Annals N. Y. Academy of Sciences* (1941), pages 77–168.

In the *J. Biol. Chem.*, **133,** 737 (1940), Rittenberg and Foster give details of their procedure for the determination of amino acids (and fatty acids) by isotopic dilution Another principle for the determination of amino acids is introduced by Bergmann and Stein, *J. Biol. Chem.*, **128,** 217 (1939); Moore and Stein, *Ibid.*, **150,** 113 (1943).

The gasometric determination of carboxyl groups in amino acids, by the use of ninhydrin, is described by Van Slyke, Dillon, MacFadyen and Hamilton, *J. Biol. Chem.*, **141,** 627, 671 (1941).

* "Knowledge of the shapes of protein molecules is far less advanced than that of their sizes," writes Edsall. "Nevertheless, there is abundant evidence that some proteins, such as hemoglobin and insulin, do not deviate very far from the spherical shape. Others, like silk fibroin, keratin, myosin and collagen are extremely elongated fibers. Still others, such as zein and certain antibody globulins of horse serum, are far from spherical but by no means fibrous."

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The interesting question of the form of glutamic acid obtained from malignant tumors has received much attention. See, for example, Anon., *J. Am Med. Assoc.*, **114**, 589 (1940); Graff, Rittenberg and Foster, *J. Biol. Chem.*, **133**, 745 (1940); Chibnall, Rees, Williams and Boyland, *Biochem. J.*, **34**, 285 (1940); Arnow and Opsahl, *Science*, **91**, 431 (1940).

A few typical preparations of amino acids are the following (the first three deal with the isolation of the amino acids from a protein and the others are synthetic methods): Bergmann and Niemann, *J. Biol. Chem.*, **118**, 301 (1937), Mazur and Clarke, *Ibid.*, **123**, 729 (1938), McCoy, Meyer, and Rose, *Ibid.*, **112**, 283 (1935) (α-amino-β-hydroxybutyric acid); Windus and Marvel, *J. Am. Chem. Soc.*, **52**, 2575 (1930) (methionine); Patterson and du Vigneaud, *J. Biol. Chem.*, **111**, 393 (1935) (homocystine); and Dunn, Smart, Redemann, and Brown, *J. Biol. Chem.*, **94**, 599 (1931) (glutamic acid); Buc, Ford and Wise, *J. Am. Chem. Soc.*, **67**, 92 (1945) (β-alanine); Albertson and Archer, *Ibid.*, **67**, 308 (1945) (histidine); Snyder, Cannon, Andreen and Peters, *Ibid.*, **64**, 2082 (1942), **66**, 513 (1944) (methionine and homocystine); Snyder and Smith, *Ibid.*, **66**, 350 (1944) (tryptophan).

Several miscellaneous articles of interest are by Borek and Clarke, *J. Biol. Chem.*, **125**, 479 (1938) (canavanine); Meyer, *Symp. Quant. Biol.*, **6**, 91 (1938) (glycoproteins); Behrens, *J. Biol. Chem.*, **141**, 465 (1941) (preparation of *l*-alanine from *dl*-alanine by the action of *d*-amino acid oxidase); Fruton, Irving and Bergmann, *Ibid.*, **133**, 703 (1940) (preparation of *d*(−) glutamic acid from *dl*-glutamic acid); Chargaff, *Ibid.*, **142**, 491 (1942), *Advances in Protein Chemistry* **1**, 1 (1944) (lipoproteins); Nicolet and Shinn, *Ibid.*, **142**, 139 (1942) (hydroxyglutamic acid); Winnick and Greenberg, *Ibid.*, **137**, 429 (1941) (use of optical rotation in the study of protein hydrolysis).

For the isolation of peptides as salts of aromatic acids, see Stein, Moore and Bergmann, *J. Biol. Chem.*, **154**, 191 (1944).

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For microbiological methods for the determination of amino acids, see Kuikon, Norman, Lyman, Hale and Blotter, *J. Biol. Chem.*, **151**, 615 (1943); **157**, 395 (1945); McMahan and Snell, *Ibid.*, **152**, 83 (1944).

The chemistry of that important industrial product, nylon, is given by Fuson, Connor, Price and Snyder, in their *Organic Chemistry* (1941), p 133; and by Fuson and Snyder, *Organic Chemistry* (1942), p. 140.

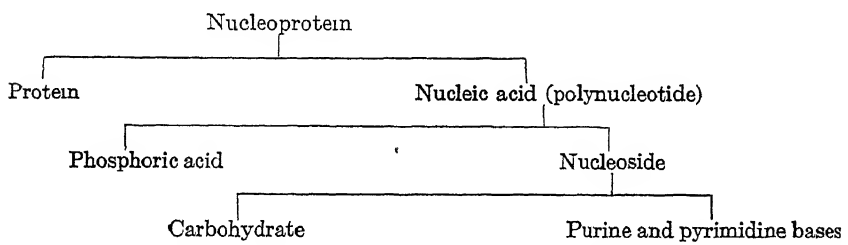
CHAPTER 5

NUCLEOPROTEINS. NUCLEIC ACIDS

NUCLEOPROTEINS are essential constituents of the nuclei of animal and plant cells. They are present in bacteria and in viruses, many of the latter being, apparently, pure nucleoprotein.

A nucleoprotein may be regarded as *any* protein attached to nucleic acid. The linkage may be of a primary (non-polar) or salt-like (polar) variety. In some cases, as in nucleohistone and in nucleic acid joined to prolamine, the nucleic acid can be separated by the addition of neutral salt. Here the linkage between nucleic acid and the protein is probably salt-like in character. Where the protein is of a more complex kind, such simple separation of nucleic acid and protein is not always possible.

The hydrolysis of nucleoprotein yields a series of successive products:



The nucleic acids, as Kossel pointed out, fall into two groups: one group is of a type derived from yeast (plant nucleic acid), and the other is represented by that obtained from the thymus gland (animal nucleic acid). All nucleic acids so far examined, no matter from what source, tend to resemble either the one group or the other. On complete hydrolysis, the two types of nucleic acid yield the following products:

Yeast nucleic acid (ribonucleic acid)	*Thymonucleic acid* (desoxyribonucleic acid)
1. Phosphoric acid	1. Phosphoric acid
2. *d*-Ribose	2. *d*-2-Desoxyribose
3. Adenine	3. Adenine
4. Guanine	4. Guanine
5. Cytosine	5. Cytosine
6. Uracil	6. Thymine

Both contain phosphoric acid and both have two purines (adenine and guanine) and one pyrimidine (cytosine) in common. They differ in the sugar component (*d*-ribose, on the one hand, and *d*-2-desoxyribose, on the other) and in the nature of one pyrimidine (uracil, in the one case, and thymine, in the other). The structure of these substances will be discussed presently.

It was supposed, for a time, that ribonucleic acid was exclusively a plant product, whereas desoxyribonucleic acid belonged to the animal world. This is not true. Ribonucleic acid has been isolated from the pancreas, for example, and desoxyribonucleic acid has been identified in plants. Furthermore, it is now pretty definitely believed that desoxyribonucleic acid is found in the *nuclei* of plant *and* animal tissues, whereas ribonucleic acid is confined to the cytoplasm.

The Importance of Nucleoproteins.—The evidence is constantly increasing that the nucleoproteins are closely associated with the chromosomes of cells, and, as we shall presently see (p. 80), with the characteristics of a number of viruses.

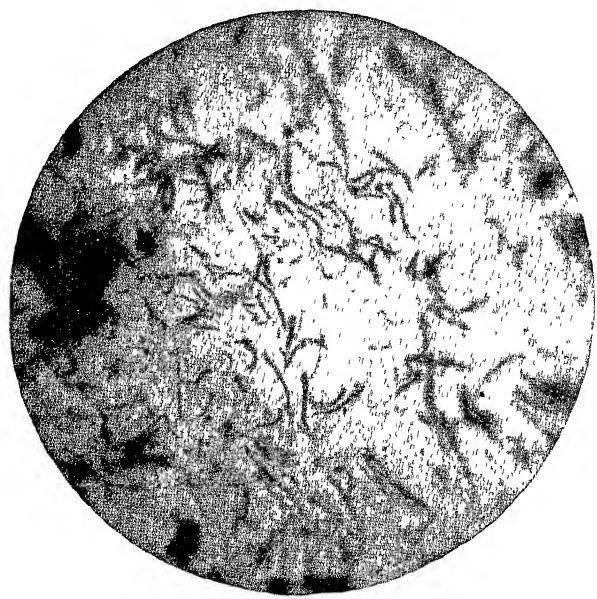


Fig. 10.—Photomicrographs of chromatin threads, isolated from nuclei of blood cells of the carp by action of a high-speed mixer. Threads stained in acetocarmine. 900 times. [Mirsky and Pollister, *Biological Symposia*, **10**, 247 (1943)].

The nucleus of the cell has an affinity for basic dyes. The material in the nucleus which can be easily stained is called *chromatin* (see Fig. 10).

During cell division discrete units appear in the nucleus, and more particularly in the part where the chromatin is found. These "units" are the *chromosomes*, "the carriers of the hereditary characteristics of the cell." The number of these "characteristics" is greater than the number of chromosomes, a fact which led to the belief that these chromosomes could be further subdivided into smaller units, each unit "carrying" a single "characteristic." It is these units which are the *genes*.

Relatively enormous chromosomes are found in the salivary glands of insect larvae—big enough, indeed, to be dissected by hand (Fig. 11).

"The great accumulation of desoxyribonucleic acid in the chromosome," writes Mirsky, "strongly suggests that these substances are

either the genes themselves or are intimately related to the genes."* Stedman and Stedman [*Nature*, **152**, 267 (1943)] claim to have isolated from the nuclei, particularly fish sperm, not only a histone (or protamine) and desoxyribonucleic acid, but a new type of protein which represents the major constituent of the nucleus. This protein, the authors claim, constitutes the chromatin of the nucleus and, hence, forms the principal component of the chromosome.

Nucleic acid in the nucleus of the cell (in chromosomes, for example) are found joined to protein, such as a histone or a protamine. The nucleic acid—protein combination can often be extracted with 1 *M* sodium chloride solution and precipitated with physiological saline (0.9 per cent NaCl).

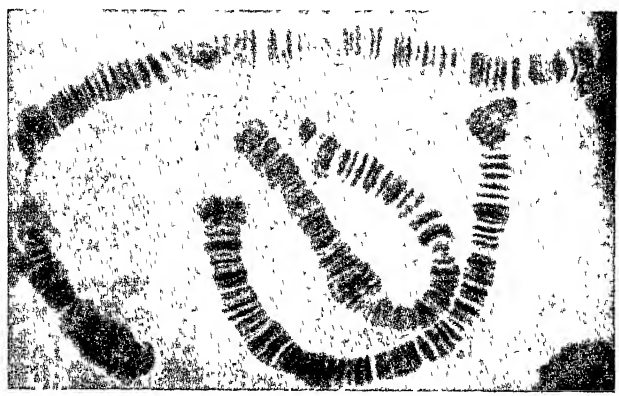
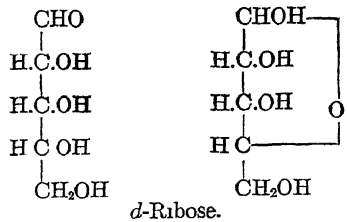


Fig. 11.—Chromosomes from a cell in the salivary gland of an insect larva. [*Monthly Science News* (Britain), June, 1943.]

Hydrolysis of Nucleic Acid.—The nucleic acid can be hydrolyzed by heating at 115° C. with very dilute ammonia for one hour. Under these conditions, a mixture of four *nucleotides* is obtained, each nucleotide consisting of a combination of phosphoric acid, a sugar and a purine (or pyrimidine) derivative. This hydrolysis can also be accomplished by the use of an appropriate enzyme—ribonuclease (obtained from beef pancreas) for ribonucleic acid. When, however, nucleic acid is heated at 180° C. with fairly dilute ammonia for three and one-half hours, phosphoric acid is split off and glycosides known as *nucleosides* are formed. The nucleoside is combination of carbohydrate and a purine or pyrimidine derivative. Hydrolysis with acid separates yeast nucleic acid into its fundamental components (phosphoric acid, a sugar, two purine and two pyrimidine bases).

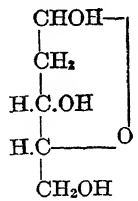
The Structure of the Two Sugars.—It has been pointed out that the nucleic acid from yeast yields *d*-ribose and that the acid from the thymus yields *d*-2-desoxyribose. Methylation studies and subsequent hydrolysis and oxidation [in which one of the products was dimethoxysuccinic acid, $COOH.(CH.OCH_3)_2.COOH$] led Levene and Tipson to propose a furanose structure for the ribose component:

* See appendix, p. 565.



d-Ribose.

From the hydrolytic products of nucleic acid obtained from thymus, Levene isolated a sugar with the formula $C_5H_{10}O_4$, which further examination and subsequent synthesis proved to be *d*-2-desoxyribose:



d-2-Desoxyribose.

The Crystalline Nucleoprotein Obtained from Mosaic-Diseased Tobacco Plants.—This important discovery, relating a protein to such diseases as measles, yellow fever, the common cold, rabies, and several mosaic and yellow diseases of plants deserves discussion.

Viruses are submicroscopic,* infectious entities which are capable of causing disease in man, animals, plants, insects, and bacteria. In the past they have been characterized by their invisibility, by their ability to pass filters capable of holding back all ordinary living things, and by their inability to multiply or reproduce in the absence of living cells. However, because viruses may reproduce under certain conditions, because they are specific in that certain viruses occur or cause disease only in certain hosts, because they may change and adapt themselves to new conditions, and because of the lasting immunity which usually follows most virus diseases, practically all of the workers in the virus field choose to regard viruses as invisible living organisms.

A study of tobacco-mosaic virus, a virus which was discovered in 1892 and was the first of these agents to be described, was undertaken by Stanley in 1935. A high-molecular weight, crystalline protein possessing the properties of tobacco-mosaic virus was isolated from Turkish tobacco plants diseased with this virus. The following facts have since been brought out: The virus appears to be a conjugated protein containing about 95 per cent protein and 5 per cent nucleic acid. This nucleic acid belongs to the *ribonucleic* acid variety.

Bushy stunt virus appears to contain 83 per cent protein and 17 per cent of a nucleic acid similar to that found in tobacco-mosaic virus.

* Viruses range in size from about 10 mμ—slightly smaller than some protein molecules—to about 300 mμ, which is somewhat larger than certain accepted living organisms (Stanley).

Tobacco ring spot virus contains 40 per cent nucleic acid, which is the highest percentage yet found in a virus.

An analysis of the protein constituent of tobacco-mosaic virus shows the presence, among others, of tyrosine, tryptophan, proline, arginine, phenylalanine, serine, threonine and cystine.* Glycine appears to be absent.

The molecular weight of tobacco-mosaic virus is about 60,000,000.

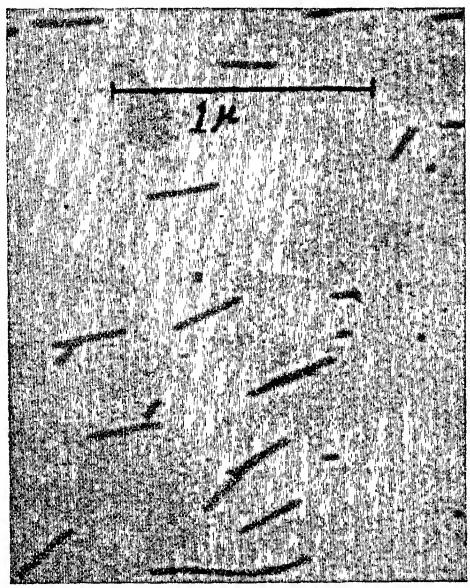


Fig. 12.—Tobacco virus molecules. Micrograph of molecules of tobacco-mosaic virus taken by means of the RCA electron microscope. Magnification × 34,000. (Anderson and Stanley, *J. Biol. Chem.*, **139**, 338.)

These viruses, nucleoprotein in composition, can perpetuate themselves by reproduction within, *and only within*, certain specific living cells.†

Some idea of the size of the molecules of the tobacco-mosaic virus may be obtained from Figs. 12 and 13.‡

* Ross reminds us that the specific properties of many biologically active proteins appear to be dependent upon the presence of sulfhydryl groups (R-SH) or disulfide linkages (R-S-S-R). The physiological effect of insulin appears to depend upon the presence of the disulfide linkage. The disulfide state appears to be the active one in a number of enzymes (phosphatase and amylase). Free sulfhydryl groups may be essential for the activity of such enzymes as urease, papain, lipases and several dehydrogenases. The activity of several principles in the posterior lobe of the pituitary may be dependent upon sulfhydryl groups.

† Stanley emphasizes the similarities in properties between viruses and genes. In size and in composition (nucleoprotein), both resemble one another. Both reproduce within certain specific living cells. Both may undergo mutations, spontaneously or as a result of irradiation, changes which are reproduced in subsequent generations. But so far, unlike viruses, it has not been possible to isolate and study genes *in vitro*.

‡ The electron microscope, which makes use of electrons instead of rays of light, and magnetic or electrostatic fields instead of glass lenses, gives magnifications up to 100,000 diameters. This means that the electron microscope is from 50 to 100 times more powerful than the strongest optical microscope.

Fig. 13.—Mixture of tobacco-mosaic viruses with normal rabbit serum. The contaminating bacterium serves to give a good idea of the relative size of the molecules of virus. Electron micrograph with magnification × ca 37,000. (Anderson and Stanley, *J. Biol. Chem.*, **139**, 344.)

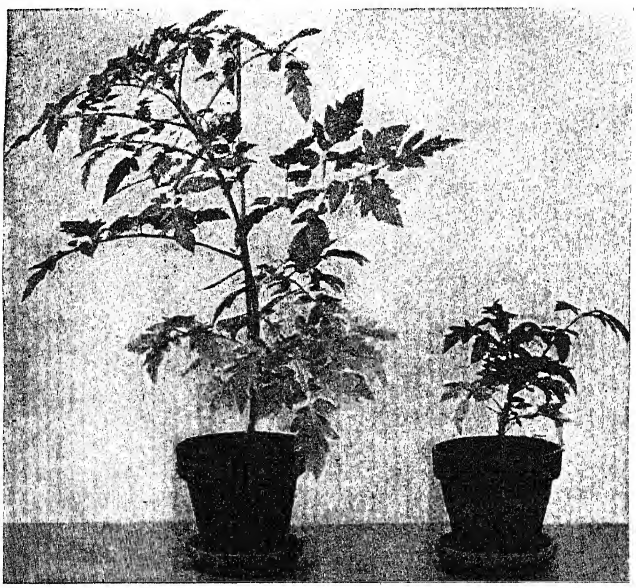


Fig. 14.—Left-hand, healthy tomato plant; right-hand, bushy stunt-diseased tomato plant of the same age. (Photograph by J. A. Carlile.) (Stanley, *J. Biol. Chem.*, **135**, 452.)

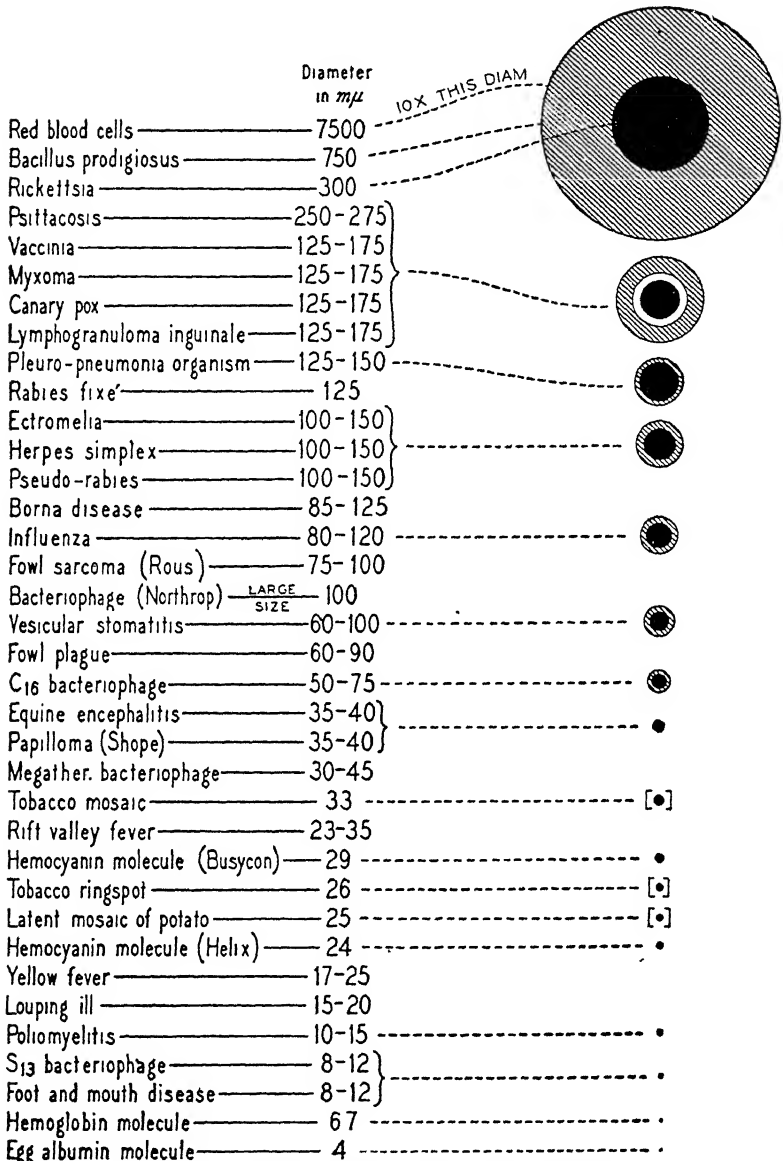


Fig. 15.—A chart showing the relative sizes of several selected viruses including bacteriophages, as compared to those of the red blood cells, *Bacillus prodigiosus*, rickettsia, pleuropneumonia organism, and protein molecules. (Stanley, *American Naturalist*, **72**, 112.)

The effect on growth of a variety of plant virus, the bushy stunt virus, is shown in Fig. 14.

In a later chapter (Chap. 6), some details are given concerning the preparation of crystalline enzymes which are proteins. Stanley's original method of isolating the crystalline protein having virus activity may interest the reader.

The injured plants are cut, frozen, ground, and the juice pressed out. The juice is adjusted to *p*H 6.7, filtered through a layer of celite (a filter aid), and 30 per cent by weight of ammonium sulfate is added to the filtrate. The precipitate is dissolved in water, the solution adjusted to *p*H 7 and the precipitation with ammonium sulfate is repeated. The final precipitate is dissolved in water and the solution adjusted to *p*H 4.5, which precipitates the active protein. The precipitate is again dissolved in water, the solution adjusted to *p*H 7, and the protein in the filtrate is crystallized by adding sufficient saturated

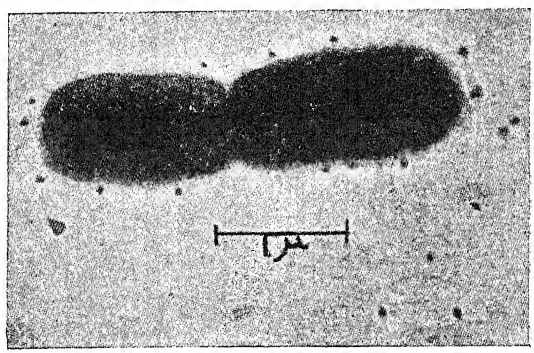


Fig. 16.—Bacteriophage particles, α strain, attached to cells of Escherichia coli. Reduced from an electron micrograph with a magnification of 17,500 diameters. (Mudd and Anderson, *J. Am. Med. Assoc.*, **126**, 561.)

ammonium sulfate to cause a slight cloudiness, then sufficient 10 per cent glacial acetic acid in one-half saturated ammonium sulfate to adjust the *p*H to 5.5, and finally sufficient saturated ammonium sulfate to bring all the protein out of solution.

How do viruses originate, reproduce or mutate? These are questions which cannot yet be answered. However, in as far as the virus itself is considered, virus activity appears to be a property of its protein molecules. It is these protein molecules which show virus activity, the essence of which, as Stanley points out, is reproduction.

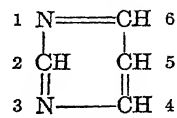
"There may be no sharp line of distinction between molecules and organisms," writes Stanley, "and the viruses may provide the transition between the two."

A chart showing relative sizes of viruses is given in Fig. 15.

Bacteriophage.—Northrop has purified bacteriophage, a filtrable virus which destroys bacteria and which possesses the common "virus" property of increasing in the presence of living cells. The bacteriophage obtained from lysed cultures of staphylococcus shows the general properties of a nucleoprotein (Fig. 16).

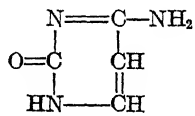
The Purines and Pyrimidines.—Aside from the two sugars and phosphoric acid, the two nucleic acids are made up of several purines and pyrimidines. To a large extent we are indebted to Emil Fischer for our knowledge of the chemistry of these substances.

Pyrimidine itself has the structure

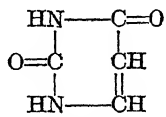


Pyrimidine.

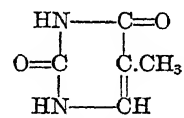
and the three pyrimidine compounds found in the two nucleic acids have the following formulas:



Cytosine.
(6-amino-2-keto-pyrimidine)

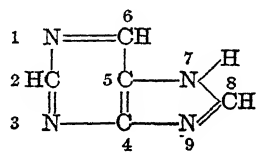


Uracil.*
(2, 6-diketo-pyrimidine)



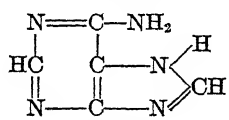
Thymine.
(2, 6-diketo-5-methylpyrimidine)
(5-methyluracil)

The purine base is

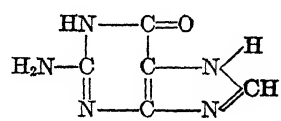


Purine.

and adenine and guanine, the two purine compounds of interest here, have the following structures:

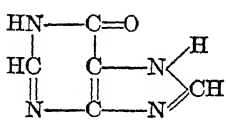


Adenine.
(6-aminopurine)

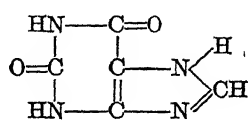


Guanine.
(2-amino-6-ketopurine)

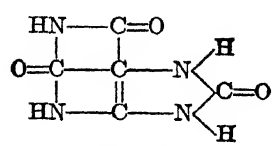
Three other purines of importance physiologically are hypoxanthine, xanthine, and uric acid.



Hypoxanthine.
(6-ketopurine)



Xanthine.
(2, 6-diketopurine)



Uric acid.
(2, 6, 8-triketopurine)
(Also called 2, 6, 8-trioxypurine)

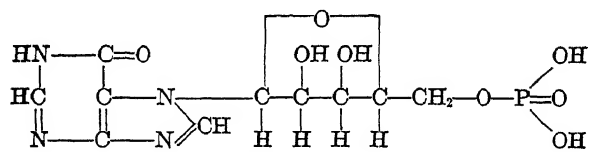
*Thiouracil, where the carbon in position 2 is attached to —SH, has been successfully used in thyroid treatment (p. 482).

The Nucleotides.—It has already been pointed out that the nucleotides are combinations of phosphoric acid, a sugar, and one of the purine or pyrimidine bases. It has also been indicated that alkaline hydrolysis of the nucleotide splits off the phosphoric acid giving a nucleoside, a combination of sugar and base. It should now be mentioned that mild acid hydrolysis splits off the base, and leaves behind the phosphoric ester of the sugar. These reactions suggest that within the molecule of a nucleotide the constituents are arranged in the following order:

Base-sugar-phosphoric acid.

Several important nucleotides are described briefly.

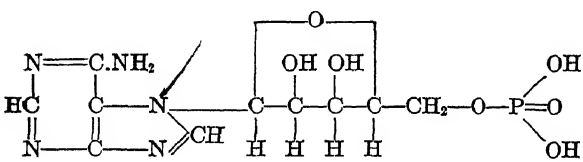
Muscle Inosinic Acid.—This nucleotide was first isolated by Liebig from beef extract, and it is an important constituent of muscle (Chap. 16). Hydrolysis of inosinic acid with hydrochloric acid yields hypoxanthine and *d*-ribose 5-phosphoric acid. Its formula has now been established:



Inosinic acid.

(7′-hypoxanthine-5-phosphoribofuranoside)

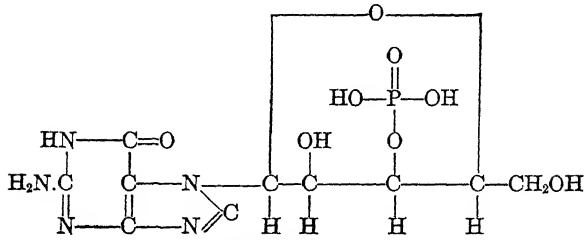
Muscle adenylic acid is another nucleotide of importance in the metabolism of muscle (Chap. 16). It can be isolated from heart muscle and from brain. An enzyme in muscle is capable of transforming adenylic acid into inosinic acid, at the same time liberating ammonia. In fact, it is now known that the difference between the two acids is that in inosinic acid the base is hypoxanthine and in adenylic acid it is adenine. Adenylic acid has the formula



Adenylic acid.

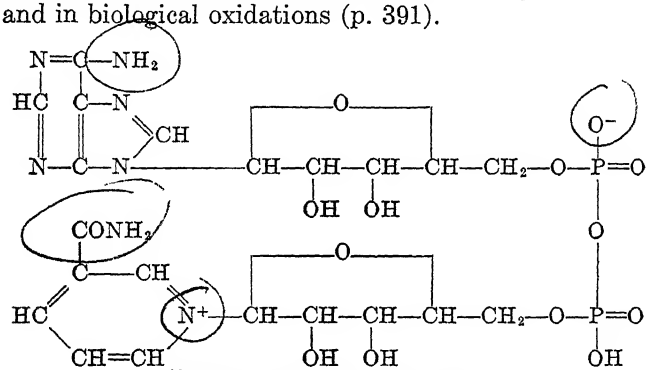
(7′-adenine-5-phosphoribofuranoside)

Guanylic acid has been isolated from animal tissues (pancreas, spleen, and liver) and from yeast. Aside from the presence of the base guanine, guanylic acid is also further characterized by the fact that the phosphoric acid-sugar combination is of the form 3-phosphoribose. The formula for guanylic acid is:



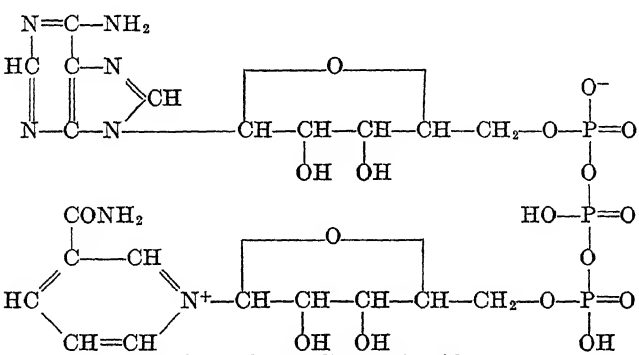
Guanylic acid.
(7′-guanine-3-phosphoribofuranoside)

Several purine or pyrimidine derivatives play an important part in biological reactions. For example, **diphosphopyridine nucleotide,** also known as *codehydrogenase I, coenzyme I and cozymase,* is an adenine-containing nucleotide and is important in yeast fermentation (p. 21) and in biological oxidations (p. 391).



Diphosphopyridine nucleotide.

Triphosphopyridine nucleotide, also known as *codehydrogenase II, coenzyme* and *coenzyme II,* differs from diphosphopyridine nucleotide

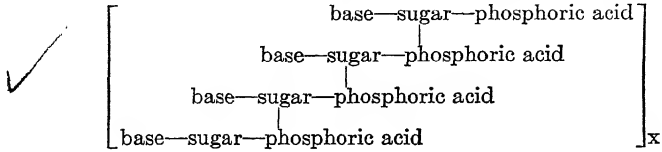


Triphosphopyridine nucleotide.

in having 3 instead of 2 molecules of phosphoric acid. It plays a rôle in biological oxidations (p. 391).

Cocarboxylase, the phosphoric acid derivative of thiamine, is a pyrimidine derivative (p. 85).

The **nucleic acids** themselves are polynucleotides which, on mild hydrolysis, yield four mononucleotides, each nucleotide containing a characteristic base. The following general formula has been proposed:



References

The chemistry of the nucleoproteins is discussed by Greenstein, *Advances in Protein Chemistry*, **1,** 209 (1944); and Levene and Bass, *Nucleic Acids* (1931). Tipson's article on nucleic acids in Harrow and Sherwin's *Textbook of Biochemistry* (1935), Chap. 7, should also be consulted. See also Loring, *Ann. Rev. Biochem.*, **13,** 295 (1944); Gulland, Barker and Jordan, *Ibid.*, **14,** 175 (1945).

For the distribution of nucleoprotein in the cell, as well as possible relationships to chromosomes, etc., see Mirsky, *Adv. Enzym.*, **3,** 1 (1943); Claude, *Biological Symposia*, **10,** 111 (1943); Mirsky and Pollister, *Ibid.*, **10,** 247 (1943); Greenstein, *Advances in Protein Chemistry*, **1,** 266 (1944).

The work on viruses, including Stanley's plant viruses, is described in many publications. See, for example, Hoagland, *Ann Rev. Biochem.*, **12,** 615 (1943); Pirie, *Adv. Enzym*, **5,** 1 (1945); Mudd and Anderson, *J. Am. Med. Assoc.*, **126,** 561 (1944); Stanley, *Science*, **101,** 185 (1945); Lennette, *Ibid.*, **98,** 415 (1943); Stanley, *Chronica Botanica*, **7,** 291 (1943); Cohen and Stanley, *J. Biol. Chem.*, **144,** 589 (1942); Price, *Science*, **101,** 515 (1945).

Some experimental studies dealing with nucleic acids or their constituents are Sevag and Smolens, *J. Biol. Chem*, **140,** 883 (1941); Davidson and Waymouth, *Biochem. J.*, **38,** 39, 375, 379 (1944); Loring and Carpenter, *J. Biol. Chem.*, **150,** 381 (1943); Buell, *Ibid.*, **150,** 389 (1943).

For a description of the electron microscope and its application, see Burton and Kohl, *The Electron Microscope* (1941); Zworykin, *Ind. Eng. Chem*, **35,** 450 (1943); Martin, *Ann. Rev. Biochem.*, **12,** 587 (1943).

The purification of bacteriophage by Northrop is described in the *J. Gen. Physiol.*, **21,** 335 (1938); and by Kalmanson and Bronfenbrenner, in the same journal, **23,** 203 (1939). A general discussion of bacteriophage, by Krueger and Scribner, may be found in the *J. Am. Med. Assoc.*, **116,** 2269 (1941).

CHAPTER 6

ENZYMES

ENZYMES are catalysts produced as a result of cellular activity "but independent of the presence of living cells in their operation" (Waldschmidt-Leitz). The name "enzyme" (from the Greek, "in leaven") is associated with the process of fermentation.

The early work dealing with enzymes was confined almost entirely to the process dealing with alcoholic fermentation. Pasteur was of the opinion that fermentation involved metabolic activity of micro-organisms within the living cell. Once the cell was destroyed no fermentation was possible. Buchner, however, overthrew this concept by showing that an active yeast juice can be expressed from yeast at a pressure which completely destroys the cells themselves.

These enzymes occur in wide abundance in all animal and plant tissues. Although a number of them have presumably been isolated in the chemically pure condition, the various enzymes are identified by their behavior toward substrates (the substances acted upon). For example, the enzyme which converts urea into ammonia is urease. We refer to the *specificity* of enzyme action, wherein a particular enzyme acts on a particular substance (substrate) or, as sometimes happens, on a group of substrates closely related chemically.

What is it that gives the enzyme its catalytic power? We do not know. Something in the structure of its protein molecule must be different from a protein molecule not endowed with such enzyme activity (egg albumin, for example). The enzyme, it is postulated by many, has some "active center," and it is with this "active center" that the substrate combines, forming an enzyme-substrate combination which can be dissociated (p. 105). The substrate in contact with the "active center" becomes "activated" itself; which means that it can undergo chemical reactions of one kind or another.

A classification and description of the various enzymes now follows:

Esterases*

(*a*) *Pancreatic Lipase.*—This enzyme is present in the pancreas and hydrolyzes fats into fatty acid and glycerol (p. 225). Lipase is also found in gastric juice (p. 221).

(*b*) *Liver Esterase.*—This enzyme, present in liver, hydrolyzes esters of simple alcohols and is an ester- rather than a fat-splitting enzyme.

(*c*) *Ricinus Lipase.*—This enzyme is found in the seeds of the castor bean and is similar to pancreatic lipase.

* By general agreement, enzymes are so named that they end in "ase." Enzymes not named in accordance with this rule were well known before the nomenclature was adopted.

(*d*) *Chlorophyllase.*—This is an enzyme present in green plants and hydrolyzes chlorophyll *a* into phytol ($C_{21}H_{39}OH$) and ethyl chlorophyllide *a* (p. 207).

(*e*) *Phosphatases.*—These enzymes are present in various tissues and hydrolyze various esters of phosphoric acid. The phosphatases, of which there seem to be quite a number, are divided into two groups: alkaline phosphatase, with an optimum *p*H of from 8 to 10, found in intestinal mucosa, kidneys, liver, etc., and acid phosphatase, with an optimum *p*H of from 4 to 5, present in blood serum, etc.

(*f*) *Azolesterases.*—This name has been suggested for enzymes which hydrolyze nitrogen-alcohol esters. The best-known example is cholinesterase, the enzyme which hydrolyzes acetylcholine (p. 538).

Proteinases and Peptidases

(*a*) *Pepsin*.—This enzyme is present in the stomach and hydrolyzes proteins to peptones (?) (p. 219). Pepsin can also act on synthetic substrates much simpler in composition than proteins (p. 92). The enzyme has been crystallized (p. 97) *

(*b*) *Trypsin.*—This enzyme is present in the pancreas and hydrolyzes proteins to peptides and amino acids (p. 224). Like pepsin, trypsin can act on substrates much simpler, chemically, than proteins (p. 93); and like pepsin, trypsin has also been crystallized (p. 98).*

(*c*) *Erepsin.*—This substance undoubtedly represents a mixture of enzymes (p. 224) of the peptidase variety (p. 91).

(*d*) *Rennin.*—This enzyme is present in the stomach and acts on the casein of milk, converting it into paracasein, which, in the presence of calcium ions, forms a milk clot (p. 221).

(*e*) *Papain.*—This enzyme is found in the milky juice of the melon tree (papaw) and in plant cells in general. It is similar to trypsin in its action and has also been isolated in crystalline form (Balls).

(*f*) *Bromelin.*—This enzyme is present in pineapple and is similar to papain.

(*g*) *Cathepsin.*—This enzyme is found in various animal cells.

There are several cathepsins, and they are believed to play important roles in syntheses and hydrolyses within the cell (p. 101).

Cathepsin, bromelin, and papain are known as the "catheptic enzymes" or "papainases." They are all three activated by HCN, H_2S, and —SH compounds. These activators are for the most part reducing substances. The "papainases" are inactivated by mild oxidation and reactivated by reduction.‡

* The crystalline material as originally prepared is not homogeneous and apparently contains more than one protein and even some nonprotein material. Furthermore, judging by their action on synthetic substrates (of the type shown on p. 92), swine pepsin differs from salmon pepsin. Both varieties, however, will hydrolyze the common proteins.

† During the course of the isolation of crystalline trypsin, Northrop and Kunitz isolated another protein-splitting enzyme, **chymotrypsin** (p. 98) which, unlike crystalline trypsin, also clots milk.

‡ Anson believes that cathepsin is not activated by cysteine, but that the enzyme is accompanied by a peptidase which is activated.

Bergmann, Fruton and coworkers are of the opinion that cathepsin as well as

(*h*) *Ficin.*—This enzyme is found in the milk sap of the fig tree. It digests proteins at about *p*H 5. It is not activated by —SH compounds.

(*i*) *Aminopeptidase.*—This is an enzyme present in the intestine and acts on polypeptides containing a free amino group. (There are probably several aminopeptidases.)

(*j*) *Carboxypeptidase.*—This is an enzyme found in the pancreas and acts on polypeptides containing a free carboxyl group. It has been crystallized (Anson). (The essential difference between *i* and *j* is the place of rupture in the peptide linkage.)

(*k*) *Dipeptidase.*—This enzyme, present in intestinal juice, hydrolyzes dipeptides. Since both aminopeptidases and carboxypeptidases split dipeptides, the existence of a separate group of dipeptides is open to question.

Even though the picture is admittedly incomplete, the following table (Table 9), prepared by Bergmann, Fruton and their coworkers, represents a marked advance in our knowledge.*

Amidases (these act on carbon-nitrogen linkages):

(*a*) *Urease.*—This is an enzyme which is present in leguminous plants (soy and jack beans) and converts urea into ammonia. It has been isolated in crystalline form (p. 96).

(*b*) *Arginase.*—This enzyme is found in liver and converts arginine into ornithine and urea (p. 357).†

(*c*) *Purine Amidases.*—These represent a group of enzymes, present in the liver, which deaminize purines. The enzyme adenase is an example.

Phosphorylases.—These are enzymes which can decompose polysaccharides as well as bring about the synthesis of the latter. For example, a phosphorylase obtained from muscle converts hexose-1-phosphate (p. 322) into a polysaccharide. The reaction is a reversible one. An x-ray diffraction pattern of this polysaccharide shows it to be similar in structure to starch obtained from plants.

In contrast to this, when heart or liver phosphorylase is used in place of the one obtained from muscle, the polysaccharide obtained resembles glycogen.

Carbohydrases

(*a*) *Sucrase* (saccharase, invertase).—This enzyme is present in animal and plane tissues and hydrolyzes sucrose into glucose and fructose (p. 4).

Maltase and *lactase*, found with sucrase in the small intestine (p. 226), hydrolyze maltose and lactose respectively.

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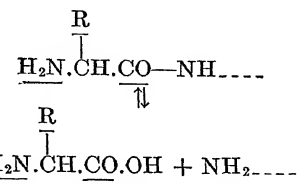
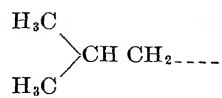
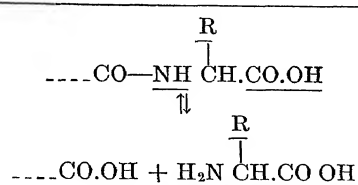
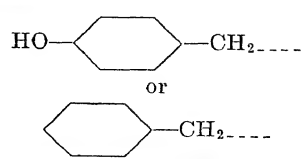
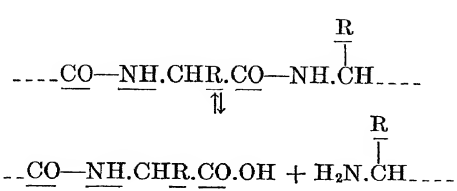
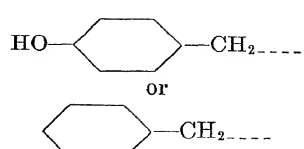
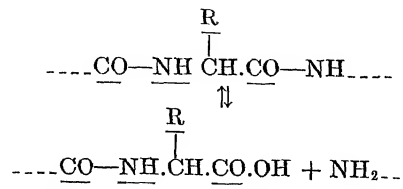
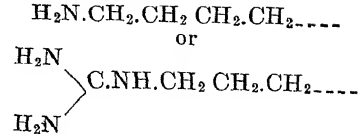
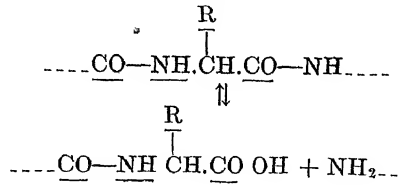
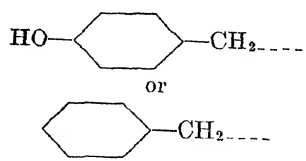
papain exist in two inactive forms (α and β forms). The α form is not activated by HCN, but may be converted into the β form which is then activated by HCN. Furthermore, the activation and inactivation of the β form can be accomplished without the mutual transformation of SS and SH groups and without the occurrence of reduction and oxidation processes.

* As ordinarily used, the proteinases are enzymes which act on proteins and the peptidases are enzymes which act on polypeptides. However, as the table shows, both types act on relatively simple synthetic substrates.

† There is evidence to show that maximum activity with arginase is obtained only in the presence of Mn^{++} ions.

TABLE 9.—SUMNER AND SOMERS, *Classification of Proteolytic Enzymes (after Bergmann) Enzymes* 1943 (Academic Press).

Enzyme.	Requisite groups in "backbone" of substrate and mechanism of catalyzed reaction.*	Requisite groups in the side chain of the substrate R =
	1. Peptidases	
A. *Aminopeptidases, e.g.,* Leucine-aminopeptidase from intestinal mucosa, amino-peptidase from spleen and kidney (Cathepsins III), etc.		
B. *Carboxypeptidases, e.g.,* Carboxy-peptidase from pancreas, carboxy-peptidases from spleen and kidney (Cathepsins IV), etc.		
	2 Proteinases	
A. Pepsin and pepsinases from spleen and kidney (Cathepsins I).		

B. Trypsin and trypsinases from spleen and kidney (Cathepsins II).		
C. Chymotrypsin.		

* The underlined portions indicate the groups within the "backbone" which are considered indispensable.

(*b*) *Emulsin.*—This enzyme is present in bitter almonds and hydrolyzes the glucoside amygdalin into glucose, benzaldehyde and hydrogen cyanide.

(*c*) *Amylases.*—These enzymes are found in plant and animal tissues and hydrolyze starch and glycogen into maltose. The ptyalin found in saliva and the amylase present in pancreatic juice are examples (pages 213, 225).

Oxidases*

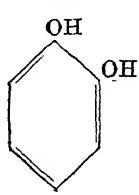
(*a*) *Dehydrogenases.*—These are enzymes, found in many tissues (muscle, for instance) which bring about oxidations by the removal of hydrogen from substances (p. 387). For example, succinic dehydrogenase converts succinic acid into fumaric acid (p. 390).†

(*b*) *Catalase.*—This enzyme is found in plant and animal tissues (liver, for example) and catalyzes the decomposition of hydrogen peroxide into water and molecular oxygen (p. 397). Sumner has obtained the enzyme in crystalline form and states that it is a combination of a protein with an iron-pyrrole (heme) compound (compare with hemoglobin, p. 258).

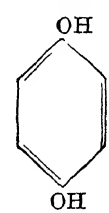
(*c*) *Peroxidases.*—These enzymes are present in many tissues (for instance, the spleen and horseradish) and they transfer peroxide oxygen to oxidizable substances (p. 398).

(*d*) *Tyrosinase.*—There is much confusion regarding this enzyme. Such various oxidation processes as the darkening of the potato on exposure and the formation of melanin (p. 366) are said to involve tyrosinase. The enzyme catalyzes the oxidation of various phenolic compounds (phenol, catechol, *p*-cresol, tyrosine, pyrogallol, etc.).‡

(*e*) *Laccase.*—This enzyme is found in plants and in bacteria and oxidizes both cathechol and hydroquinone types of compounds.



Catechol.



Hydroquinone.

The enzyme is a copper-protein compound (p. 399).

(*f*) *Indophenol oxidase* (*cytochrome oxidase*).—This enzyme is present in various tissues and catalyzes the oxidation of a mixture of *p*-phenylenediamine and α-naphthol to indophenol (p. 399).

(*g*) *Uricase.*—This enzyme is found in liver and kidney and oxidizes uric acid to allantoin (p. 380).

(*h*) *Luciferase.*—This enzyme is present in fireflies and acts on luciferin to produce light. Here some of the energy from the catalytic

* Much of Chap. 19 is devoted to a discussion of these enzymes.

† Coenzymes are also needed (p. 95).

‡ Tyrosinase has also been called catecholase, cresolase, polyphenol oxidase, phenolase, etc.

oxidation of an organic compound of unknown composition (luciferin) is emitted as light (bioluminescence).

Properties of Enzymes.—Enzymes show the properties of colloidal systems. A number of the enzymes which have been crystallized are protein in nature; these seem to be of the type which catalyze hydrolytic reactions (like pepsin or urease). In the field of oxidations, where oxidizing enzymes are active (Chap. 19), we find that such enzymes are usually made up of a protein "carrier" ("träger"), of high molecular weight, joined on to some dialyzable and therefore simpler group (the "prosthetic" group). It would seem that in such cases the reaction within the substrate molecule is due to the prosthetic group, but the velocity and the specificity are largely due to the "carrier."

With some exceptions among the vegetable lipases, enzymes are soluble in water. They are also soluble in dilute glycerol (which is, incidentally, an excellent preserving medium) and dilute alcohol, and are readily precipitated by strong alcohol and by saturating the solution with ammonium sulfate. They are very sensitive to temperature changes and to changes in *p*H. These enzymes are, as a rule, active not far from body temperature (37°−38° C.). Their optimum *p*H activity is sometimes in the neighborhood of (chemical) neutrality (*p*H 7.0), although in the case of pepsin the optimum *p*H is considerably lower. Being colloids (or associated with colloids) they are nondiffusible and can often be partially purified by dialysis; although here difficulties may arise due to the possible removal of some **coenzyme,** a substance necessary for the complete activity of many enzymes. For example, dialysis of yeast juice removes phosphorus compounds without which zymase is powerless to convert sugar to alcohol.

Enzymes may be present in the cell in an inactive form (a **zymogen**); some activator, or some autocatalytic process, may convert the zymogen into the active enzyme. It was believed for a time that the pepsin of the stomach appears first in the form of pepsinogen (a zymogen), and that the hydrochloric acid converts the pepsinogen into pepsin; but it is known now that pepsinogen is changed autocatalytically into pepsin at an acid *p*H.

The Preparation of Active Extracts of Enzymes.—Maceration or autolysis of the cellular material is usually a preliminary step. Of course, with digestive juices such as saliva or gastric juice, even this preliminary operation is unnecessary. The enzymes being, as a rule, soluble, can be extracted with water or dilute glycerol. This at once yields an active but very crude extract, the activity being recognized and estimated by the effect of the extract on a suitable substrate and the extent of the change produced. A crude extract of sucrase from yeast may be obtained in this way. This crude extract readily hydrolyzes sucrose into invert sugar (glucose + fructose).

In order to purify the enzyme, one or more methods may be adopted. The enzyme may be precipitated with some suitable precipitant (such as ammonium sulfate) and then redissolved, removing the excess ammonium sulfate and other impurities which may be

present by submitting the solution to dialysis. Or the method of adsorption and elution, so extensively practiced by Willstätter, may be adopted. Here the enzyme is adsorbed on alumina gel or kaolin, depending upon the weak basic or acidic properties of the enzyme, and then suitably removed (eluted) from the adsorbing material (by dilute acid, alkali, phosphate, etc.). This method in many cases yields very active extracts, but it has not so far led to the actual isolation of the enzyme itself. Salt precipitation, in the hands of Sumner and

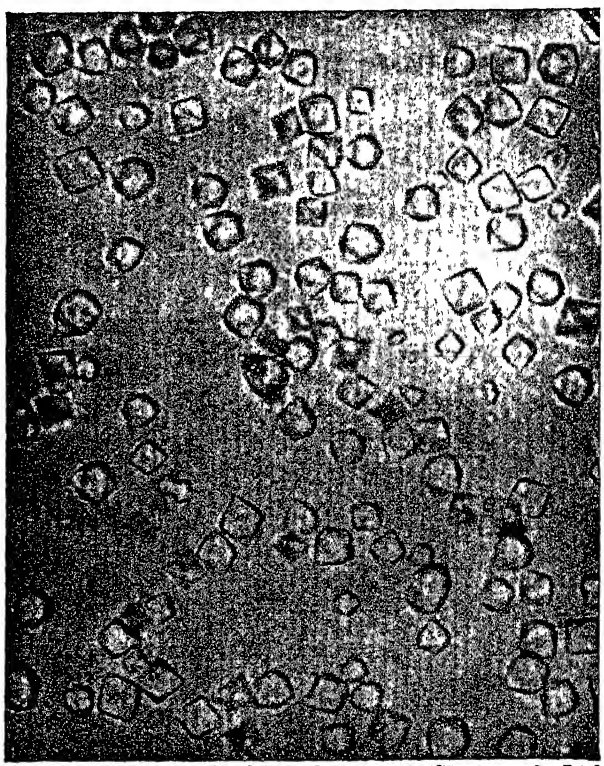


Fig. 17.—Urease crystals magnified 728 diameters. (Sumner, *J. Biol. Chem.*, **69**, 436.)

of Northrop and his associates (Kunitz, Herriott) has led to more definite results.

The Isolation of Enzymes.—Beginning with the work of Sumner in 1926, when the crystallization of urease was first announced, a number of workers in the United States and elsewhere have succeeded in isolating enzymes in the crystalline form. Some twenty enzymes have been crystallyzed. The conviction is growing that such crystalline products represent pure enzymes. These enzymes have the properties of proteins.

Sumner obtained urease in crystalline form in a surprisingly simple way. He extracted finely powdered, fat-free jack bean meal with 31.6 per cent acetone and allowed the material to filter by gravity in the

ice-chest. After standing overnight, the filtrate was centrifuged and the precipitate of crystalline urease was stirred with cold 32 per cent acetone and centrifuged again. The crystals were finally dissolved in water and allowed to crystallize. Fig. 17 shows the appearance of the crystals of urease.

These octahedral crystals are 730 times more active (in decomposing urea into ammonia) than the most active commercial jack bean meal. The crystals are soluble in water, the solution coagulates on heating and gives the biuret, xanthoproteic, Millon, Hopkins-Cole and ninhydrin tests. The enzyme is completely precipitated by saturating the solution with ammonium sulfate.

The most striking work in this field has been done by Northrop and Kunitz, who not only have isolated pepsin and trypsin and several

Fig. 18 —Pepsin crystals. (Northrop.)

enzymes associated with them, but have presented evidence that the activity is really a property of the protein molecule. The guiding principle is stated by Northrop in one of his papers: "Nearly all attempts to isolate enzymes have been carried out with relatively small quantities of material in rather dilute solution. Adsorption methods have been extensively used. If enzymes really are proteins, these are not favorable procedures for their isolation, since proteins are extremely unstable in dilute solution and are easily injured by adsorption on surfaces. The attempt to isolate pepsin was undertaken from the point of view of protein chemistry, using only those conditions under which proteins are relatively stable; that is, in concentrated solutions and at relatively low temperature."

The purification was carried out by precipitation with magnesium sulfate at various hydrogen ion concentrations and temperatures. (See Appendix p. 565 and Fig. 18.)

From the pancreas, by the use of acid extracts, and by fractional precipitation with ammonium sulfate, Kunitz and Northrop obtained four proteins in crystalline form. One of these was trypsinogen, an

inactive enzyme (zymogen) which can be converted to trypsin (the active proteolytic enzyme) by a substance called "enterokinase" in the pancreatic juice. In this manner the trypsin itself was obtained in crystalline form (Fig. 19). The other two proteins, chymotrypsinogen and chymotrypsin, separated from the trypsin and trypsinogen by fractional precipitation with ammonium sulfate, were further characterized. The chymotrypsinogen could not be activated by enterokinase; obviously, it differed from trypsinogen. However, traces of trypsin converted this protein into a powerful proteolytic enzyme which differed from trypsin in that it clotted milk. This enzyme was given the name "chymotrypsin." (See Appendix, p. 566 and Fig. 20.)

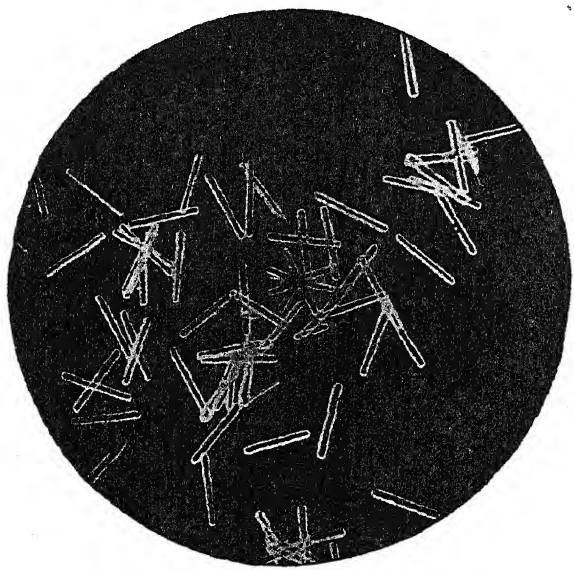


Fig. 19.—Trypsin crystals. (Kunitz and Northrop.)

Kunitz has shown that chymotrypsin in solution gradually undergoes an irreversible transformation into new enzymes, two of which he has isolated. The new modifications exhibit the same enzymatic behavior as chymotrypsin itself, but they differ from the parent substance in molecular weight, crystalline form, stability in acid and alkali, and solubility, among other things.

Trypsinogen can be converted into the active enzyme trypsin by merely allowing an aqueous solution of crystalline trypsinogen to stand. The change can be accelerated by the addition of ammonium sulfate or magnesium sulfate, enterokinase (p. 223), or trypsin. The best *p*H range for these changes is from 7.0 to 9.0. At a *p*H less than 4.0, there is practically no formation of trypsin.

Pure trypsin gradually digests itself at *p*H 7.0 to 9.0, which means that at this *p*H much of the trypsin which is formed is destroyed. A kinase probably somewhat similar to enterokinase, which Kunitz discovered in the mold of the genus Penicillium, rapidly converts crystal-

line trypsinogen into trypsin at *p*H 2.5 to 4.0. At this *p*H trypsin is quite stable.

The mold kinase acts like a typical enzyme. The process follows the course of a catalytic unimolecular reaction, the rate of formation of trypsin being proportional to the concentration of the kinase. However, the ultimate amount of trypsin formed is independent of the concentration of the kinase.

The crystalline pepsin isolated by Northrop is about five times as active (in hydrolyzing proteins) as the commercial product. One gm. of the crystalline material will digest 50,000 gm. of boiled egg white

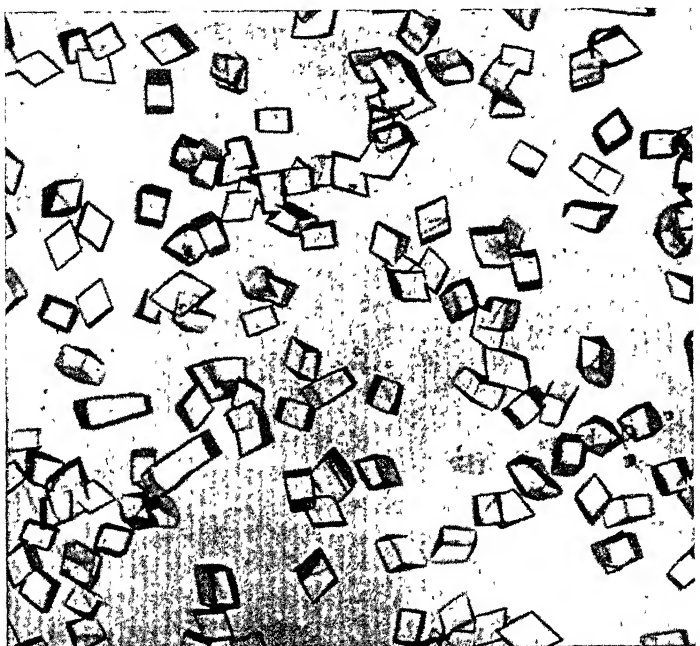


Fig. 20.—Chymotrypsin crystals (crystallized at *p*H 4.0). (Kunitz.)

in two hours and will clot 100,000 liters of milk or liquefy 2000 liters of gelatin in about the same size. The crystalline pepsin and trypsin have been recrystallized many times without showing any change in composition and properties. Acetylation studies tend to show that the acetyl groups which decrease the activity are probably attached to the hydroxyl group of tyrosine.

Certain enzymes will be discussed, either again or for the first time, in subsequent chapters (Chaps. 10, 19).

Activators and Inhibitors.—There seems to be no sharp distinction between "activators" and "coenzymes." At one time an "activator" was defined as a substance which converts an inactive into an active enzyme (like the conversion of pepsinogen into pepsin by hydrochloric acid). A "coenzyme," on the other hand, was regarded as a substance which accelerated the action of an enzyme (like the cozymase of yeast). The tendency now is to regard nonspecific substances, like

hydrochloric acid, which increase enzymic activity as "activators" and specific substances, like cozymase, which act similarly, as "coenzymes." Mention has also been made of enterokinase as an activator or coenzyme which converts trypsinogen into trypsin. The presence of salt is known to accelerate the action of amylase in hydrolyzing starch. Cathepsin and papain are activated by hydrogen sulfide and sulfhydryl compounds in general, and by hydrogen cyanide. The activities of papain and bromelin are also influenced by glutathione, and it appears that cathepsin is affected by ascorbic acid (vitamin C).

On the other hand, there are inhibitors as well as activators. A well-known example of the action of an inhibitor is the influence of blood serum on trypsin. Crystalline urease inhibits the coagulation of milk by rennin. Generally speaking, proteolytic enzymes have a destructive effect; and, in a general way, heavy metals exert a toxic influence on enzymes.

Kunitz and Northrop isolated from beef pancreas a polypeptide in crystalline form which acts as an inhibitor toward trypsin by forming a chemical compound with it.

The Synthesis of Proteins.—Not only are proteins hydrolyzed in the digestive tract, but they are synthesized and hydrolyzed in the various cells of the body. These reactions *in vivo* are all due to the activities of enzymes. While one of the simplest of biochemical laboratory procedures is to hydrolyze proteins by an appropriate proteolytic enzyme, the reverse process, that of synthesizing proteins from amino acids, has not yet been accomplished outside of the body. By purely chemical means, using his method of polypeptide synthesis, Emil Fischer built up a polypeptide containing 18 amino acids which, it was believed, showed certain resemblances to native proteins. Again, stomach extracts added to a concentrated solution of the products of peptic hydrolysis gave a precipitate which suggested the presence of protein. This precipitate was given the name of "plastein." This "plastein" could be formed even more readily when peptic digests of egg albumin were concentrated, pepsin added, and the mixture kept at 37° C. and a *p*H of 4.0.

While, strictly speaking, the work of Northrop and his associates has little to do with the synthesis of protein, yet his success in converting inactive protein material into active (enzymatically active) protein should not be overlooked at this point. Slight changes in an inactive protein sometimes result in the production of an active enzyme. Sometimes the reaction is autocatalytic; in this case the enzyme forms itself from inert proteins.

Acetylating pepsin causes an appreciable loss of peptic activity, which is subsequently recovered by the hydrolysis of the acetylated product (Herriott and Northrop). Pepsin may be denatured and inactivated by bringing the solution to *p*H 9.0 to 10.0. Part of the peptic activity can be recovered slowly by neutralizing the solution to *p*H 5.4. Warming an acid (*N*/100 HCl) solution of trypsin denatures and inactivates the enzyme. Below 30° C. the enzyme is in the active form, and at 50° C. it is 90 per cent inactivated. Trypsin here is in

equilibrium with an inactive protein, and the equilibrium is dependent upon temperature

Crystalline chymotrypsinogen, in itself completely inactive, is activated by trypsin. The rate of activation is proportional both to the concentration of the trypsin and to the concentration of chymotrypsinogen. In the course of this change, nothing is split from the chymotrypsinogen molecule. However, there is an increase of 5 amino groups per molecule. This suggests that the change from the inactive to the active enzyme involves the opening of a peptide ring.

The production of active trypsin from inactive trypsinogen brings out the importance of dealing with pure material. Amorphous trypsinogen is stable and can be activated only by enterokinase (also called "kinase," p. 223), by large quantities of trypsin or by the addition of concentrated solutions of magnesium or ammonium sulfate. After crystallization, the trypsinogen is quite unstable and is rapidly transformed into the active enzyme (trypsin) when dissolved in neutral solution. The reaction here is an autocatalytic one. The difference in behavior of the amorphous and of the crystalline material is attributed to the presence of the inhibitor in the former.

The inactive form of pepsin, pepsinogen, which is more resistant to the action of alkali than the active enzyme, has now been crystallized by Herriott and Northrop. In slightly acid solution the pepsinogen is converted to active pepsin. The reaction at *p*H 4.65 is autocatalytic, which means that it is caused by pepsin itself.

In so far as protein synthesis is concerned, Bergmann has developed a hypothesis that in this process the intracellular proteinases (the papainases) play an important part. In fact, Bergmann believes that these same enzymes are either hydrolytic or synthetic in their action depending upon "small differences in the structure of the substrate coupled with the very exactly tuned specificity of the individual intracellular enzymes." In support of this hypothesis, Bergmann points out that papain acts on two similar peptide-linked compounds in a reversible manner. The enzyme *hydrolyzes* benzoylglycine amide (1) into benzoylglycine and ammonia:

$$C_6H_5CONHCH_2CONH_2 \rightarrow C_6H_5CONHCH_2COOH + NH_3$$

and *synthesizes* benzoylglycine anilide (2) from benzoylglycine and aniline:

$$C_6H_5CONHCH_2COOH + C_6H_5NH_2 \rightarrow C_6H_5CONHCH_2CONHC_6H_5$$

(1) may be looked upon as representing an *extracellular* protein and (2) as representing an *intracellular* protein.

The Synthesis of Polysaccharides.—The action of the phosphorylases on hexoses has already been referred to (p. 25).*

* A word may here be added with regard to the importance in industries of these enzymes. The basis of the brewing industry, the production of wines and distilled liquors and the manufacture of alcohol for industrial purposes center around the use of various enzymes found in yeast cells. Bacterial enzymes are used in the manufacture of vinegar and acetic acid, butyl alcohol, acetone, lactic acid, gluconic acid, etc

As an example of the remarkable development of some of these industries,

KINETICS OF ENZYME ACTION

Since enzymes are considered organic catalysts, what is known of the laws of catalysis should apply to enzymes. The difficulty is that many of these enzymes represent colloidal substances of unknown chemical constitution, and the application of chemical dynamics becomes no easy matter. In those cases where the enzymes have been isolated they reveal themselves as proteins; and to deduce laws involving proteins as catalysts is apparently a more difficult matter than to deduce them when the catalyst is a simple inorganic substance. Even in the latter case there are many problems which await solution.

The equilibrium point of a chemical reaction is more rapidly reached in the presence of an enzyme capable of influencing the particular reaction. The enzyme is apparently unchanged at the end of the reaction and contributes no measurable energy to it. Irrespective of whether all enzymes are proteins or not, all of them are colloids, or so intimately associated with colloids that they cannot be separated from them. This may involve heterogeneous as well as homogeneous reactions; it may involve adsorption phenomena as well as reactions in solution. The enzymes are often inhibited in their activity by increasing the quantity of substrate; a phenomenon which has led to various theories of intermediate enzyme-substrate formation. The enzymes are further inhibited in their activity by the accumulation of the products of the reaction, the tendency here being toward a reversal of the reaction. They are very sensitive to changes in temperature and to *p*H changes; and they are easily "poisoned" by heavy metals, etc.

Effects of Increasing the Concentration of the Enzyme.—According to the law of mass action, the velocity of a reaction is proportional to the concentration of the reacting substances. Assuming, for the sake of simplicity, a unimolecular reaction, involving but one type of molecule, the velocity of such a reaction can be expressed as

$$v = \frac{dx}{dt} = k(a - x)$$

in which a is the initial concentration, x the change in concentration during the time t, k is a constant (unimolecular velocity constant) and $\frac{dx}{dt}$ is the velocity of the reaction. By integration we get

$$k = \frac{1}{t} ln \frac{a}{a - x} *$$

Plotting $a - x$ against t, we get a curve as shown (Fig. 21):

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it may be mentioned that as far back as 1935, 10 million pounds of citric acid were produced by the action of *Aspergillus niger* on sugar. The importation from Sicily of citric acid (made from lemons) has entirely ceased.

The retting of flax, the "curing" of tobacco, the production of pickles and sauerkraut, the ripening of cheeses, etc., are other examples of enzyme action, the enzymes being present in the various microorganisms.

* See, for example, Daniels, *Mathematical Preparation for Physical Chemistry* (1928), p. 132.

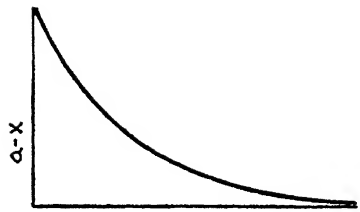

Fig. 21.

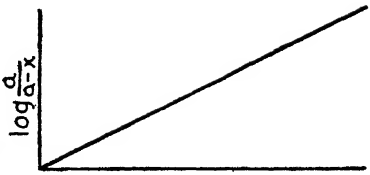

Fig. 22.

Figs. 21 and 22.—[Hitchcock, *Physical Chemistry for Students of Biology and Medicine* (2d ed.), Charles C. Thomas, Publisher.]

Plotting the log of $\frac{a}{a-x}$ against t gives us a straight line (Fig. 22).

These curves are typical of reaction velocity curves for a unimolecular reaction.

A simple example of a monomolecular reaction is the inversion of cane sugar by acid:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow C_6H_{12}O_6 + C_6H_{12}O_6$$

Strictly speaking, this is a bimolecular reaction because of the presence of water; but the change in its concentration during the reaction is negligible. The acid in the reaction is the catalyst. The results of an experiment are given in Table 10.

TABLE 10.—REACTION COURSE OF CANE SUGAR INVERSION BY H IONS (Wilhelm).

Time (t) minutes.	Angle of rotation, deg.	$K = \frac{1}{t} \log_{10} \frac{a}{a-x}$
0	+46.75	
45	+38.25	0.001 34
120	+26.00	0.001 38
240	+11.50	0.001 40
450	− 4.50	0.001 47
630	−10.00	0.001 39
∞	−18.70	... ..

By substituting an enzyme for the acid, we get results shown in Table 11.

TABLE 11.—SACCHARASE QUANTITY AND REACTION VELOCITY (Hudson).

Relative saccharase concentration.	Time, minutes.	Transformation (per cent) with initial concentration of sugar of		
		4.55 per cent.	9.09 per cent.	27.3 per cent.
2.00	15	73.2	45.3	11.2
1.50	20	73.2	44.8	11.2
1.00	30	72.9	45.3	11.5
0.50	60	72.9	45.2	11.4
0.25	120	73.1	45.2	10.9

In this case the table shows the proportionality between the concentration of enzyme and the reaction velocity.

It would seem from Northrop's work that deviations from this rule must be attributed to the presence of impurities which inhibit the reaction. For example, difficulties had always been encountered with proteolytic enzymes; but Northrop showed that in very dilute solution (where the effect of the inhibitor is minimized) and particularly by using "pure" trypsin, results in accord with theory are obtained (Fig. 23).

Northrop also showed that where the enzyme is inhibited by the products of the reaction, the direct proportionality between enzyme

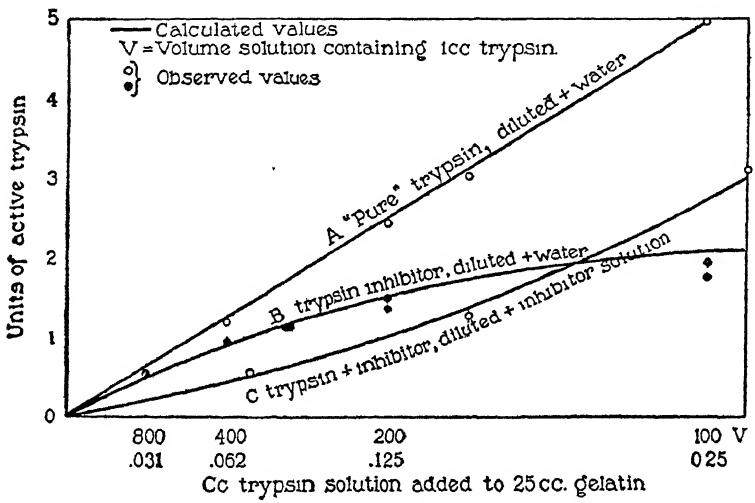

Fig. 23.—The influence of the presence of inhibitor on the concentration activity curve of trypsin. Curve A, "pure" trypsin diluted with water. Curve B, mixture of trypsin and inhibitor diluted with water. The ratio of trypsin to inhibitor is therefore constant. Curve C, mixture of trypsin and inhibitor diluted with a solution of inhibitor of the same concentration as was present in the trypsin solution. The concentration of inhibitor is therefore constant in this experiment (Northrop, *Harvey Lectures*, Williams and Wilkins Co , Publishers.)

concentration and reaction velocity is modified in accord with the **Schütz rule:** the amount of protein hydrolyzed is proportional to the square root of the concentration of the enzyme:

$$x = k\sqrt{ET}$$

where T = time (Fig. 24).

Effect of Concentration of Substrate.—Table 11 shows very clearly how the reaction velocity is influenced by the concentration of the substrate. Within a given time, a definite amount of saccharase (invertase) will hydrolyze a larger percentage of sucrose when the solution is dilute than when it is concentrated. This is illustrated in Fig. 25.

To explain such retardation, Bayliss introduced his adsorption theory, wherein the colloidal enzyme particles became "saturated" and "a further increase in concentration will not result in more adsorp-

tion and therefore in no increase in the rate of reaction." Michaelis has suggested another explanation. He believes that there is an intermediate compound formation between enzyme and substrate upon

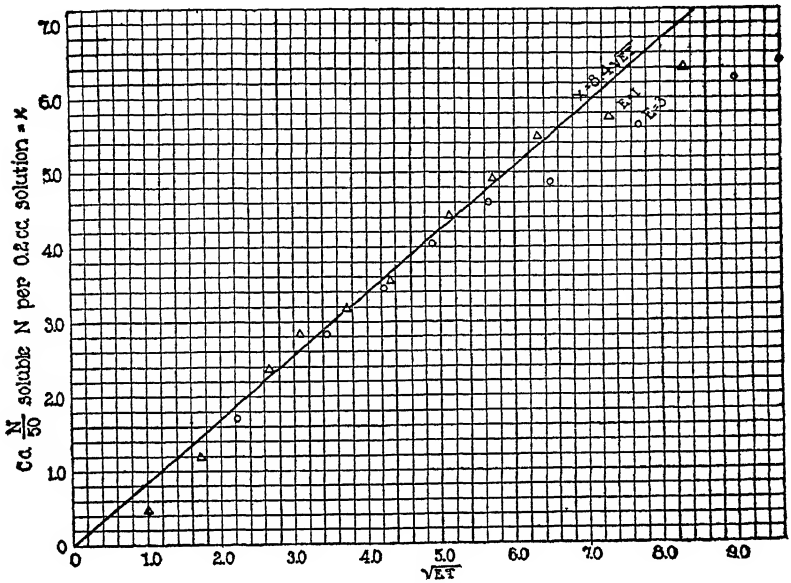

Fig. 24.—Rate of digestion of casein solution plotted against the square root of the enzyme concentration × the time in days. (Northrop, *Harvey Lectures*, Williams and Wilkins Co., Publishers)

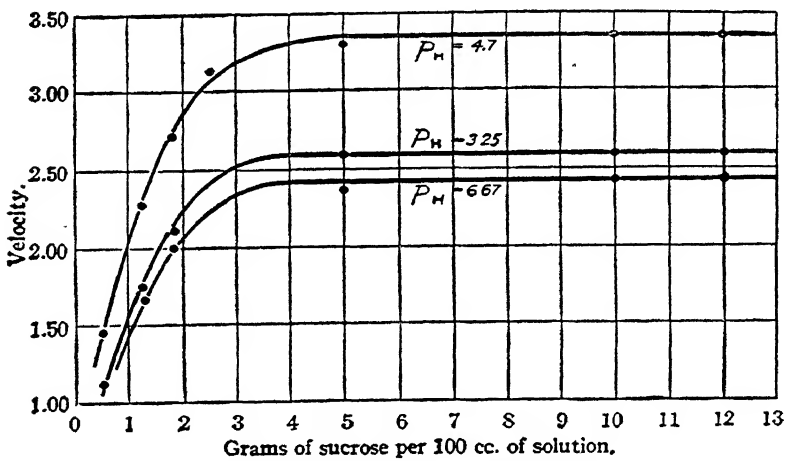

Fig. 25.—Effect of substrate concentration on reaction velocity, illustrating the limiting or saturation value at higher concentrations. Experiments with sucrose and invertase. (Nelson and Bloomfield, *J. Am. Chem. Soc.*, **46**, 1027.)

which the velocity of the reaction depends. The enzyme combines with the substrate to form an unstable intermediate compound, which may then dissociate into its two components, or decompose with the formation of free enzyme and the products of the reaction.

The view of Michaelis has recently received support from the work of Stern on the action of catalase on monoethyl hydrogen peroxide From a study of the absorption spectrum, Stern finds that an intermediate compound is formed and that it rapidly breaks down into free enzyme and reaction products. Keilin and Mann, studying the action of peroxidase on hydrogen peroxide by visual spectroscopy, also conclude that an intermediate compound is formed which rapidly decomposes in the presence of a suitable "acceptor."

In at least one instance, that of pepsin, Northrop explains enzymic peculiarities on the basis that the reaction is between the ionized protein and the free enzyme. In the presence of acid (the pepsin acting in an acid medium), the protein forms an ionized protein salt which varies an amount depending upon the *p*H of the solution. The pepsin,

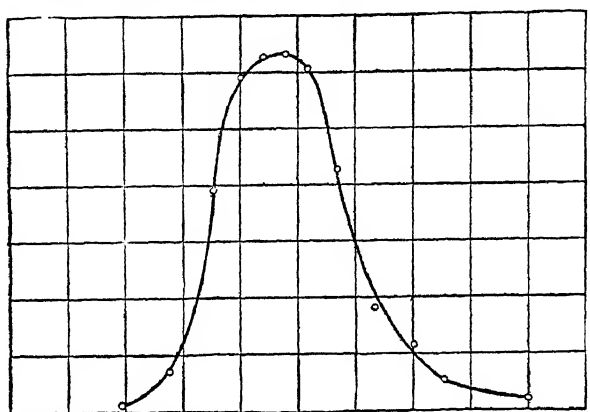

Fig. 26.—Showing the influence of hydrogen ion concentration on the activity of wheat flour amylase. (Reprinted by permission from Gortner, *Outlines of Biochemistry*, John Wiley and Sons, Inc., Publishers.)

on the other hand, is present as free pepsin, negatively charged, and as pepsin in combination with the products of hydrolysis of the protein.

Effect of Hydrogen Ion Concentration.—As has already been intimated, the activity of the enzyme is very much dependent upon the hydrogen ion concentration of the solution. Figure 26, representing the action of amylase on starch under varying *p*H conditions, illustrates the situation.

It will be noticed that the optimum *p*H (the *p*H giving maximum enzymic activity) lies between 4 and 5.

Another curve of this type we owe to Michaelis and Davidsohn (Fig. 27). Here we are dealing with the action of invertase (saccharase) on sucrose. The optimum *p*H is at 4.5. Both on the acid and the alkaline side there is a rapid decrease in activity. Between *p*H 4.5 and 9 the shape of the curve resembles the ionization curve of a weak acid; which suggests that invertase itself might be such a weak acid (within these *p*H limits), dissociating thus:

$$\text{Invertase acid} \underset{\text{Acid}}{\rightleftharpoons} \text{Invertase anion} + H^+$$

and that it is the unionized portion which catalyzes the hydrolysis of sucrose. The shape of the curve from *p*H 2 to 4.5 resembles the ionization curve of a weak base. Michaelis believes that invertase is amphoteric.

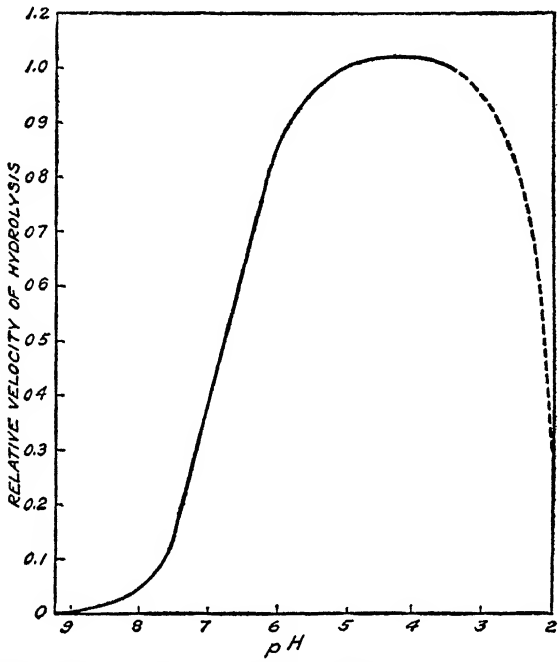

Fig. 27.—Relationship between the *p*H and activity of a yeast invertase preparation. (Michaelis and Davidsohn, *Biochem. Z.*, **35**, 405.)

Table 12, taken from the book by Waldshmidt-Leitz (see references at the end of the chapter), gives the *p*H optimum of a number of the enzymes.

Table 12.—*p*H Optimum for Several Enzymes.

Enzyme	*pH optimum*
Lipase (pancreas)	8
Lipase (stomach)	4–5
Lipase (castor oil)	4.7
Pepsin	1.5–1.6
Trypsin	7.8–8.7
Urease	7.0
Invertase	4.5
Maltase	6.1–6.8
Amylase (pancreas)	6.7–7.0
Amylase (malt)	4.6–5.2
Catalase	7 0

Northrop has compared the rate of digestion of proteins at different hydrogen ion concentrations with the titration curves of the proteins themselves. The results are given in Fig. 28. The relationship between the rate of digestion of the protein and the concentration of ionized protein is very close. "The assumption," writes Northrop,

"that only the negative protein ions are attacked by trypsin and only the positive ions are attacked by pepsin will serve quite well, at least as a first approximation."

The Effect of Temperature.—Enzymic reactions are influenced by temperature changes in much the same way that chemical reactions in general are influenced by them. Very approximately, for an increase in temperature of 10° C. the velocity of the reaction is doubled or tripled. Between 20° and 30° C., then, the temperature coefficient (Q_{10}, or k_{30}/k_{20}) for many enzymic reactions is between 2 and 3. However, it must be remembered that enzymes are very susceptible to heat, and that temperatures from 50° C. and up for any length of time

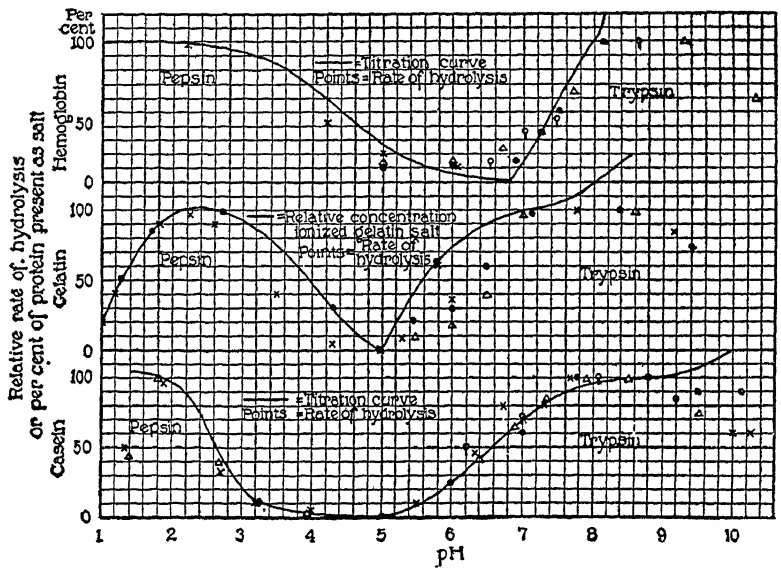

Fig. 28.—Comparison of titration curves to rate of digestion of various proteins. (Northrop *Harvey Lectures*, Williams and Wilkins Co , Publishers.)

(and the time factor is very important) very rapidly destroy them. As the temperature is increased beyond body temperature, two forces come into play: the rate of destruction of the enzyme versus the increase in the rate of transformation of the substrate; and at comparatively high temperatures the first completely overshadows the second.

References

A general book on enzymes is by Sumner and Somers (1943). The monograph by Haldane (1930) deals more with the kinetics of enzyme reactions. Tauber, in his *Enzyme Technology* (1943), takes up the important problem of enzymes as applied to industry.

Several important reviews are the following: Mann and Lutwak-Mann, *Ann. Rev. Biochem.*, **13,** 25 (1944) (non-oxidative enzymes); Herriott, *Ibid.*, **12,** 27 (1943) (proteolytic enzymes); Piman, *Adv. Enzym.*, **4,** 68 (1944) (carbohydrases, etc.); Greenberg, Winnick, Lineweaver and Jansen, *Ann. Rev. Biochem* , **14,** 31, 69 (1945).

In vol. **1** (1941) of the *Adv. Enzym.*, may be found an article on proteinase by Bergmann and Fruton; and another on catalase by Sumner The extensive work by

Bergmann and Fruton dealing with proteolytic enzymes as specific agents in the formation and breakdown of proteins, is reviewed by Fruton in *Symp. Quant. Biol.*, **9**, 211 (1941).

References to enzymes more specifically active in biological oxidations are given at the end of Chap 19 At this stage it may be sufficient to mention two volumes: *Mechanisms of Biological Oxidations* (1940) by Green, and *Respiratory Enzymes* (1942), Univ. Wisconsin Press.

Two papers by Sumner on urease are in the *J. Biol. Chem.*, **69**, 435 (1926), and **76**, 149 (1928). An interesting autobiographical sketch may be found in the *J. Chem Educ.*, **14**, 255 (1937).

An excellent review of the work of Northrop and his associates on the isolation of enzymes is by Northrop in the *Harvey Lectures* for 1934–35, Ser. 30, page 229.

Anson's crystalline carboxypeptidase is described in the *J. Gen. Physiol.*, **20**, 663 (1937). Crystalline catalase is discussed by Sumner and Dounce in the *J. Biol. Chem.*, **115**, 417 (1937), and crystalline papain by Balls and Lineweaver in *J. Biol. Chem.*, **130**, 669 (1939) and by Balls, Circular 631 (Dec., 1941), U. S. Dept. Agriculture

The synthesis of protein is the subject of articles by Wasteneys and Borsook in the *J. Biol. Chem.*, **62**, 15, 633 (1924), and **63**, 563 (1925); Cuthbertson and Tompsett, *Biochem J.*, **25**, 2004 (1931); and by Bergmann and Niemann in *Science*, **86**, 187 (1937).

The enzyme-substrate theory is discussed by Stern in the *J. Biol. Chem.*, **114**, 473 (1936), and by Northrop, *Harvey Lectures*, Ser. 21 (1925–1926), p. 71. See, also, Chance, *J. Biol Chem.*, **151**, 553 (1943).

A few further reviews are: Bergmann, *Harvey Lectures* (1935–1936), p. 37 (proteins and proteolytic enzymes); Northrop, *Physiol. Rev.*, **17**, 144 (1937) (the formation of enzymes); Nelson, *Chem. Rev.*, **12**, 1 (1933) (invertase); Linderstrom-Lang, *Harvey Lectures* (1938–1939), p. 214 (enzymic activity in cells); and Harvey, *Ann. Rev. Biochem.*, **10**, 531 (1941) (luciferase), Fruton, Ball, Bergmann, Kalckar, Meyerhof and Smythe, *Annals N. Y. Academy of Sciences*, **45**, 359 (1944) (energy relationships in enzyme actions)

A selection of individual papers on various aspects of enzyme chemistry follows:

Adams and Hudson, *J. Am. Chem. Soc.*, **65**, 1359 (1943); Adams, Richtmeyer and Hudson, *Ibid.*, **65**, 1369 (1943) (purification of invertase).

Wagreich and Nelson, *J. Am. Chem. Soc.*, **60**, 1545 (1938); Parkinson and Nelson, *Ibid.*, **62**, 1693 (1940) (on the nature of tyrosinase).

Fruton, Irving and Bergmann, *J. Biol. Chem.*, **138**, 249 (1941); **141**, 763 (1941) (proteolytic enzymes of animal tissues).

Irving, Fruton and Bergmann, *J. Biol. Chem.*, **139**, 569 (1941) (activation of intracellular proteonases).

Fruton and Bergmann, *J. Biol. Chem.*, **127**, 627 (1939); Bergmann, Fruton and Pollock, *Ibid.*, **127**, 643 (1939); Hofmann and Bergmann, *Ibid.*, **130**, 81 (1939) (specificities of pepsin and trypsin).

Greenstein, *J. National Cancer Institute*, **2**, 511 (1942) (phosphatases).

Wallerstein, *Ind. Eng. Chem.*, **31**, 1218 (1939) (commercial production of enzymes).

Irving, Fruton and Bergmann, *J. Biol. Chem.*, **138**, 231 (1941); Hofmann and Bergmann, *Ibid.*, **138**, 243 (1941) (kinetics of proteinase action).

Winnick, Davis and Greenberg, *J. Gen. Physiol.*, **23**, 275 (1940); Greenberg and Winnick, *J. Biol. Chem.*, **135**, 761 (1940) (physico-chemical properties of plant proteases).

Sizer, *Adv. Enzym.*, **3**, 35 (1943) (effect of temperature on enzyme kinetics).

CHAPTER 7

FOODS[1]

OUR foods include proteins, fats (lipids), carbohydrates, mineral salts, vitamins, water, and oxygen. Separate chapters are devoted to vitamins (Chap. 8) and water (Chap. 21), and under blood (Chap. 13) and respiration (Chap. 15) a discussion of the function of oxygen is undertaken. The changes which carbohydrates, fats, proteins, and mineral salts undergo in the body are treated in subsequent chapters (Chaps. 16, 17, 18, 21). At this stage we wish to refer to a few pertinent facts involving food and diet in general, as a preliminary to a discussion of digestion.

Over a considerable period of time the emphasis was laid on the calorific needs of the body. Calorimetric studies (Chap. 20) revealed that a normal person weighing 70 kg. expends, on an average, energy equivalent to about 3000 Calories per day.[2] The plus and minus variations are considerable, depending upon age and depending upon the amount of physical labor expended. The first column in Table 13[3] deals with calorific requirements.

For many years, foods have been analyzed for their "energy" content; and those foods high in calorific value have been preferred. While it is well recognized that the energy needs of the body must be fulfilled if health is to be maintained, the shift in emphasis has been, first, toward a better distribution within the diet of the three basic foodstuffs (protein, fat, and carbohydrate), and secondly, toward a more careful consideration of the individual components of the diet (and this would also include the mineral salts and vitamins).

The Amount of Protein in the Diet.—This problem has proved a difficult one. More than fifty years ago, Voit made an elaborate study of what the average German laborer consumes in the shape of protein. This led him to the view that the needs of the average man were in the neighborhood of 118 gm. of protein per day. Chittenden, working at Yale twenty years later, maintained nitrogen equilibrium (where the intake and the output of nitrogen are approximately equal) on as little as 45 gm. of protein per day. But the problem assumed an entirely new aspect as a result of the pioneer researches of Kossel and Fischer on the chemical constitution of the protein molecule. It became apparent that the emphasis must be placed on the amino acid content of the protein, rather than on the protein as a whole.

For example, it had been known for some time that gelatin is a "deficient" protein. That is to say, assuming the presence in the diet

[1] See also Chapters 8 and 20.

[2] For the average individual in the white collar class in America, this figure is probably somewhat high.

[3] See Chapter 20.

of optional amounts of fat, carbohydrate, etc., the use of gelatin as the sole source of protein proved disastrous.* An analysis of gelatin showed that it was deficient in a number of important amino acids. Tryptophan, valine and isolencine are entirely missing; tyrosine and cystine are present in small amounts (compare with table on p. 49).

TABLE 13.—RECOMMENDED DAILY ALLOWANCES FOR SPECIFIC NUTRIENTS.[4] Committee on Foods and Nutrition, National Research Council.

	Calories	Protein, grams.	Calcium, grams	Iron, mg.	Vitamin A,[6] I. U	Thiamin (B^1), mg [5–7]	Riboflavin, mg.	Niacin (nicotinic acid), mg.	Ascorbic acid, mg [5–7]	Vitamin D, I U.
Man (70 kg.)										
Moderately active ..	3000	70	0 8	12	5000	1 8	2 7	18	75	[9]
Very active	4500					2 3	3 3	23		
Sedentary ..	2500					1 5	2 2	15		
Woman (56 kg)										
Moderately active ..	2500	60	0 8	12	5000	1 5	2 2	15	70	[9]
Very active .	3000					1 8	2 7	18		
Sedentary...	2100					1 2	1 8	12		
Pregnancy (latter half)	2500	85	1 5	15	6000	1 8	2 5	18	100	400–800
Lactation .	3000	100	2 0	15	8000	2 3	3 0	23	150	400–800
Children up to 12 years										
Under 1 year[7]	100/kg.	3–4/kg.	1 0	6	1500	0 4	0.6	4	30	400–800
1–3 years[8]...	1200	40	1 0	7	2000	0 6	0 9	6	35	[9]
4–6 years .	1600	50	1 0	8	2500	0 8	1 2	8	50	
7–9 years	2000	60	1 0	10	3500	1 0	1 5	10	60	
10–12 years	2500	70	1.0	12	4500	1 2	1 8	12	75	
Children over 12 years										
Girls, 13–15 yrs.	2800	80	1 3	15	5000	1 4	2.0	14	80	[9]
16–20 yrs.	2400	75	1 0	15	5000	1 2	1 8	12	80	
Boys, 13–15 yrs.	3200	85	1 4	15	5000	1 6	2 4	16	90	[9]
16–20 yrs.	3800	100	1 4	15	6000	2 0	3 0	20	100	

Further Recommendations.—The requirement for iodine is small; probably about 0.002 to 0.004 milligram a day for each kilogram of body weight. . . . This need is easily met by the regular use of iodized salt, its use is especially important in adolescence and pregnancy.

The requirement for copper for adults is in the neighborhood of 1 0 to 2.0 milligrams a day. Infants and children require approximately 0 05 [mg] per kilogram of body weight. The requirement for copper is approximately one-tenth of that for iron.

The requirement for vitamin K is usually satisfied by any good diet. Special consideration needs to be given to newborn infants. Physicians commonly give vitamin K either to the mother before delivery or to the infant immediately after birth.

[4] Tentative goal toward which to aim in planning practical dietaries; can be met by a good diet of natural foods. Such a diet will also provide minerals and vitamins, the requirements for which are less well known.

[5] 1 mg. thiamin equals 333 I. U.; 1 mg. ascorbic acid equals 20 I U.

[6] Requirements may be less if provided as vitamin A, greater if provided chiefly as the provitamin carotene.

[7] Needs of infants increase from month to month. The amounts given are for approximately 6–8 months. The amounts of protein and calcium needed are less if derived from breast milk.

[8] Allowances are based on needs for the middle years in each group (as 2, 5, 8, etc.), and for moderate activity.

[9] Vitamin D is undoubtedly necessary for older children and adults. When not available from sunshine, it should be provided probably up to the minimum amounts recommended for infants.

A number of investigators next showed that growth and development—though probably not *normal* growth and development—are possible by the addition to the diet of the missing amino acids.

* "Cadet de Vaux in Paris during the French Revolution tried to persuade the poor that gelatin soup was a satisfactory and nutritious diet. The poor refused and their attitude has since been amply justified. . . . " T. F. Dixon, *Nature*, March 4, 1944.

Economically, the problem of gelatin is of importance. It is a cheap protein. It is easily obtained from tendon, cartilage, bone, and skin by boiling with water, thereby converting the collagen into gelatin.

Osborne and Mendel.—We owe to Osborne and Mendel the fundamental work on the effect in the diet of various proteins viewed in the light of their content of amino acids. Casein, despite its deficiency in glycine, is an excellent protein (Fig. 29). Apparently, glycine is an amino acid which is either not needed, or which can be synthesized by the body when needed. We know the latter view to be the correct one.*

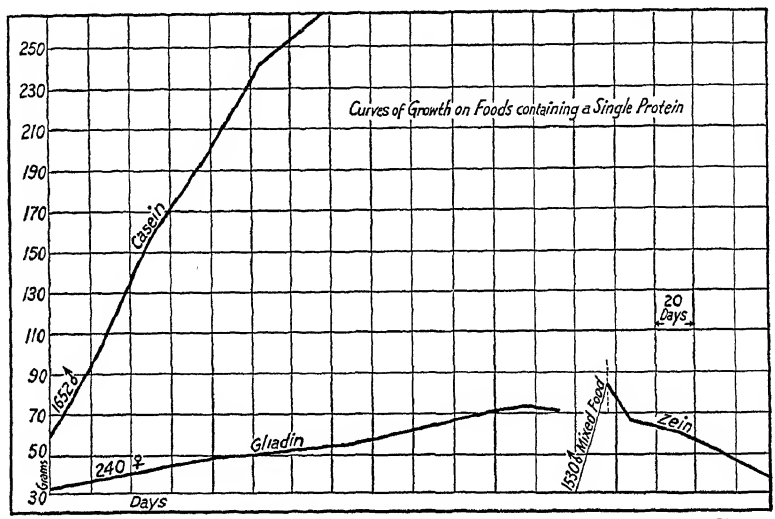

Fig. 29.—Showing typical curves of growth of rats maintained on diets containing a single protein. On the casein food (devoid of glycine) satisfactory growth is obtained; on the gliadin food (deficient in lysine) little more than maintenance of body weight is possible; on the zein food (devoid of glycine, lysine, and tryptophan) even maintenance of body weight is impossible (Osborne and Mendel, *Harvey Lectures*. Ser. 10, Williams and Wilkins Co , Publishers)

Gliadin (a protein in wheat and rye) and zein (in maize) are obviously deficient.

These experiments—and others later, such as the experiment illustrated in Fig. 30—established the indispensability of certain amino acids (such as lysine and tryptophan). It became clear that a diet devoid of such amino acids (or rather, a diet in which the proteins are deficient in such amino acids) is a deficient diet. Apparently, the synthetic abilities of the body are limited.

Rose.—The experimental method adopted by Osborne and Mendel was to feed carefully purified proteins and to supplement "deficient" proteins with the missing amino acids. Another method which has been widely adopted may be illustrated by an example. Casein is first completely hydrolyzed until a mixture of amino acids is obtained. From this mixture histidine is removed as completely as possible. The residue proves deficient. Supplementing the residue with histidine

*This is true for the rat but not necessarily for other animals.

causes recovery. Histidine is, then, an essential or indispensable amino acid (Fig. 31) (Rose; Harrow and Sherwin).

The third method, adopted by Rose, is very costly, but it is the most satisfactory method. It consists in feeding a mixture of purified amino acids, comparable in number and in relative amounts to those found in casein (which is a biologically wholesome protein). These highly purified amino acids (19 in number), added to a diet of the necessary fat, carbohydrate, mineral salts, and vitamins, were quite

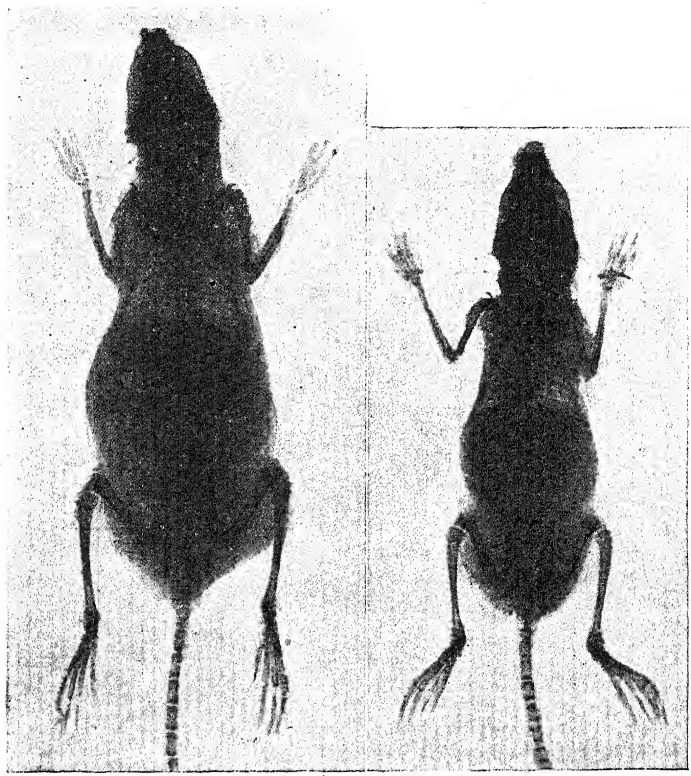

Fig. 30 *a*. Fig. 30 *b*.

Fig. 30.—Radiograph of lysine-deficient animal (30 *b*) and control (30 *a*). The animals were littermates, 10 weeks old and had been 6 weeks on their respective diets. (Harris, Neuberger and Sanger, *Biochem. J.*, **37**, 508.)

inadequate for the normal growth of young rats. This result was in striking contrast to the use of hydrolyzed casein as the source of protein: here normal growth was obtained.* Evidently, some unknown substance or substances essential to growth were present in the hydrolyzed casein and absent in the synthetic mixture of amino acids. The substitution of part of the synthetic amino acid mixture with some native protein, such as casein, improved the condition of the animals. Eventually, from the monoamino-monocarboxylic acid fraction of the hydrolyzed protein, Rose isolated a new amino acid, threonine or

* See appendix, p. 567.

α-amino-β-hydroxybutyric acid (Fig. 32). This acid is found in casein, fibrin, serum albumin, and serum globulin, among others, but hemoblobin contains little, if any.

A list of essential (indispensable) and non-essential (dispensable) amino acids (in so far as these have been studied up to the present) is given in Table 14.

Fig. 31.—Lower photograph shows a rat on a histidine-deficient diet. Upper rat received the same diet together with a histidine supplement. *Courtesy Prof. W. C. Rose and Journal of Biological Chemistry.*

Rose defines an indispensable amino acid as one which cannot be synthesized by the organism out of materials ordinarily available at a speed commensurate with the demands for normal growth.

TABLE 14.

Essential amino acids	*Non-essential amino acids*
Lysine	Glycine
Valine	Alanine
Tryptophan	Serine
Methionine	Norleucine
Histidine	Aspartic acid
Phenylalanine	Glutamic acid
Leucine	Hydroxyglutamic acid
Isoleucine	Cystine
Threonine (α-amino-β-hydroxybutyric acid)	Citrulline
Arginine*	Proline
	Hydroxyproline
	Tyrosine

* Arginine can be synthetized by the animal organism, but not at a sufficiently rapid rate to meet the demands of *normal* growth.

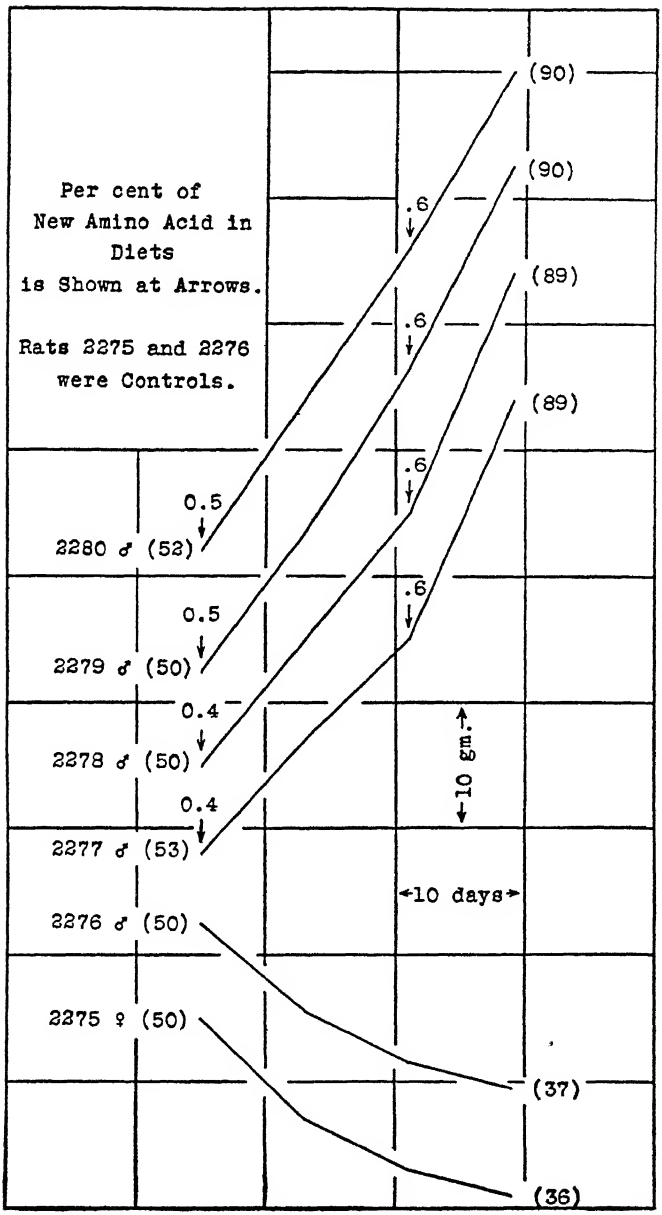

Fig. 32 —Rats 2275 and 2276 did not grow on a purified amino acid mixture The addition of the new amino acid—threonine—caused growth. (McCoy, Meger and Rose, *J. Biol. Chem.*, **112**, 283)

Another factor which has to be taken into consideration in dealing with these amino acids is their optical activity. Where the synthetic product is used, Rose gives *double* the theoretical amount to insure the presence of the *active* isomer at the desired level.

Rose finds that with the following amino acids, only the *natural* isomers promote growth: valine, leucine, isoleucine, lysine, and threonine; on the other hand, in dealing with tryptophan, histidine, phenylalanine, and methionine, it is found that both isomers promote growth.

In Table 15 the minimum quantities of essential amino acids needed for growth are given (these values represent the percentages of the natural amino acids which must be present in the rations).*

TABLE 15.—MINIMUM AMOUNT OF EACH ESSENTIAL AMINO ACID NECESSARY TO SUPPORT NORMAL GROWTH WHEN THE NONESSENTIALS ARE INCLUDED IN THE FOOD (Rose).

Amino acid	Per cent
Lysine	1.0
Tryptophan	0.2
Histidine	0.4
Phenylalanine	0.7
Leucine	0.9
Isoleucine	0.5
Threonine	0.6
Methionine	0.6
Valine	0.7
Arginine	0.2
	5.8

The experiments of Rose dealing with essential and nonessential amino acids apply to the rat. Rose has also used dogs in place of rats and finds that, in general, the response is similar. These results, however, do not necessarily apply to all other animals. For example, Almquist, using chicks in the place of rats, showed that glycine and arginine were important.

In so far as man is concerned, the experiments are still in a preliminary stage. Perhaps the most striking result (obtained by Rose) is that here histidine is dispensable. Another investigator, Holt, finds that the lack of arginine decreases the number of spermatozoa to about one-tenth. Sperm cells, it is known, contain relatively large amounts of arginine; and Holt suggests that the lack of arginine in the diet gives rise to atrophy of the spermatogenic tissue.

The clinician is familiar with the results of an inadequate protein diet over an extended period. One such result is nutritional edema, also known as war or starvation edema—a condition produced in animals when fed a low protein diet. In this condition, the plasma protein (the albumin fraction) is considerably below normal in amount (hypoproteinemia).

As showing to what extent these amino acids are the true building stones of the protein in the body, Whipple has restored the plasma protein in dogs which had previously been bled (reducing tissue and

* It would be rather rash to draw the conclusion that the non-essential amino acids have little value in nutrition. Given a set of rigid experimental conditions (for *rats*), the results just outlined may hold. But one must be careful not to draw too sweeping conclusions.

plasma protein) by feeding them protein hydrolysates—the resulting mixture of amino acids obtained after the protein has been hydrolyzed.

High and Low Protein.—In the light of such work as that of Osborne and Mendel, and of Rose, the danger of incorporating too little protein in the diet becomes apparent. The problem must be attacked, first, from the point of view of including proteins which contain all the essential or indispensable amino acids, and, secondly, from the point of view of including enough of such proteins, so that the essential amino acids may be present in sufficient amounts. Dealing in dietetics for man, with complex natural foodstuffs rather than with artificial mixtures, the answer to the problem is not an easy one. Both Sherman and Rose are of the opinion that an allowance for the adult of 70 to 75 gm. per day of mixed proteins is within the region of safety. This is approximately 1 gm. of protein per kilogram of body weight. The British Ministry of Health advocates 50 gm. of "first class protein," meaning by that protein of animal origin (milk, eggs, cheese, meat, and fish).

It is worth noticing in this connection that meat is by no means the *only* source of animal protein. Nor, if meat it must be, is there evidence that, nutritionally speaking, expensive cuts are necessarily superior to portions less expensive. And some parts of the animal, such as blood, lungs, brain and heart, richly nutritious, are often spurned.

At this stage a word should be said about the proteins in the soybean, of which *glycinin* is the most abundant. While, from the point of view of essential amino acids, animal proteins are to be preferred to vegetable proteins, the proteins of the soybean take their place somewhere in between—on the whole, better than other vegetable proteins, but probably not quite so good as animal proteins. Cooked soybean meal is superior to the raw variety. This has been explained on the basis that heat treatment makes the amino acids methionine and cystine more readily available.*

More than 90 per cent of the soybean protein is at present used for cattle feed.

The proteins in yeast closely approximate, in biological value, those found in the soybean; which means that, on the whole, they are superior to most of the proteins found in the vegetable world.

The Amounts of Fat and Carbohydrate in the Diet.—Fats and carbohydrates are essentially energy-yielding foods. If the protein intake is equivalent to 75 gm., some 300 Calories can be attributed to protein (1 gm. of protein is equivalent to 4 Calories). If the total calorific requirements is about 3000 Calories, more than 2000 Calories must be derived from the burning of fat and carbohydrate in the body. This means that the calorific equivalent of the fat plus the carbohydrate must be between 2000 and 3000 Calories. Just what proportions of fat and carbohydrate should be included is probably not important, within broad limits. This much is definite: in the absence of carbo-

* As a rule, the soybean meal on the market has already been subjected to heat treatment, since the meal is a by-product obtained during the course of the extraction of the oil—a process which involves heating.

hydrate, and when the fat alone supplies all the energy requirements, acidosis sets in. On the other hand, in the absence of fat from the diet, no matter how much carbohydrate is present, rats develop a deficiency disease (Figs. 33 and 34). This deficiency is due to the absence from the diet of highly unsaturated fatty acids of the type

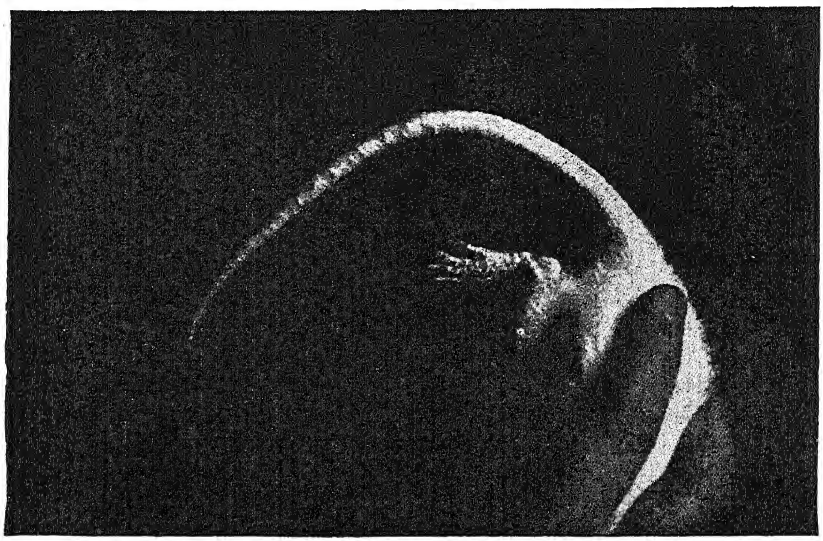

Fig. 33.—Scaly skin of hind feet and tail of rat on fat-deficient diet. (Burr, in Visscher, *Chemistry and Medicine*, University of Minnesota Press.)

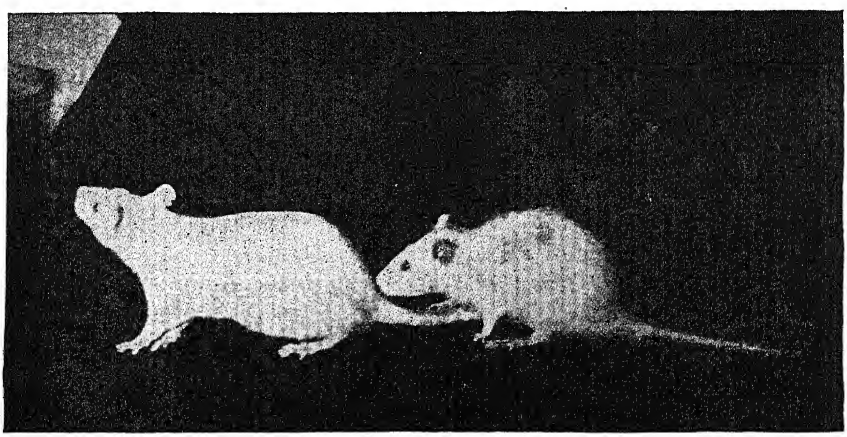

Fig. 34.—Litter mate sisters with and without fat in the diet. (Burr, in Visscher, *Chemistry and Medicine*, University of Minnesota Press.)

of linoleic acid (p. 31)—acids present in various fats. It should also not be overlooked that many fats are admixed with vitamins.

Fats almost always contain variable quantities of lipids such as lecithin. The choline present in the lecithin molecule prevents the development of fatty livers (p. 334).

Not only is fat needed in the diet for the various reasons stated,

but when used it should be preferably *fresh*. A rancid fat is unpalatable and has a destructive action on other foods, particularly vitamins A and E (see next chapter). The fat in this condition may even be somewhat toxic. Sometimes the extent to which a food can be preserved will depend upon the condition of the fat present—the more rancid the fat the more rapid the deterioration of the food mixture.

Apparently, both carbohydrate and fat must be incorporated in the diet. Many dietaries contain about four times as much carbohydrate as fat (by weight). A typical dietary might be composed of 75 gm. of protein ($75 \times 4 = 300$ Calories*), 80 gm. of fat ($80 \times 9 = 720$ Calories) and 400 gm. of carbohydrate ($400 \times 4 = 1600$ Calories); with a total calorific value of 2620.

Inorganic Elements. [Later on a chapter (Chap. 21) is devoted to this topic.]—The common inorganic elements found in animal tissues include calcium, magnesium, sodium, potassium, sulfur, phosphorus, chlorine, iron, and iodine. The spectroscope has revealed traces of many other elements. Some of them, such as copper, seem as essential for the normal development of the animal as do the amino acids themselves. These inorganic elements play various rôles: as components of skeletal structures; as cellular constituents; as regulators of body neutrality; etc.

Acid-Base Balance of Foods.—In Chap. 15 we shall learn how body neutrality is maintained. At this stage it may be mentioned that foods burnt in the body may also influence the acid-base balance. For example, meat and eggs are foods which when oxidized in the body give rise to acids. On the other hand, many fruits and vegetables are potential base-formers. The sulfur and phosphorus of proteins—abundant in meat and eggs—are oxidized to sulfuric and phosphoric acids. On the other hand, the organic acids of fruits are often, though not always, oxidized to carbonates.

The nutritive value of some common foods is given in the Appendix (p. 543).

Vitamins.—See Chap. 8.

VARIOUS FOODS

Milk.—It is generally agreed that no one natural food compares with milk in its "protective" capacity. No other food so well protects the individual from possible deficiencies in the diet. For the past decade particularly, nutritional experts have been advocating a more liberal consumption of milk; and this applies not altogether to the poorer section of the community, though, of course, the need is most urgent here.

Aside from its protein, fat, and carbohydrate supply, milk is rich in calcium, phosphorus, and vitamin A. It also contains appreciable quantities of other vitamins and minerals.

One standard set by a state (N. Y.) insists that milk must contain 11.5 per cent of solids, 3 per cent of fat, and not more than 88.5 per

* These are large calories, or Kg. calories. See Chap. 20.

cent of water. As compared with this standard, the actual average composition of milk is as follows (in per cent): casein 3.0, fat 3.7, milk sugar 4.7, albumin 0.4, ash 0.7, other constituents 0.06, and water 87.3. Present in traces, and yet of extreme importance, are a number of the vitamins (Chap. 8).

Milk Fat.—The milk fat represents, in the main, a mixture of a number of fats containing as a rule, saturated fatty acids. Associated with these fats are vitamins A and (to a lesser extent) D Commercially, the value of milk depends upon its fat ("butter-fat") content, for much of cream and butter and cheese represents "fat."

The fats in milk and the quantities (in percentages) are as follows: butyrin, $C_3H_5(COOC_3H_7)_3$, 3.8; caproin, $C_3H_5(COOC_5H_{11})_3$, 3.6; caprylin, $C_3H_5(COOC_7H_{15})_3$, 0.5; caprin, $C_3H_5(COOC_9H_{19})_3$, 1.9; laurin, $C_3H_5(COOC_{11}H_{23})_3$, 7.4; myristin, $C_3H_5(COOC_{13}H_{27})_3$, 20.2; palmitin, $C_3H_5(COOC_{15}H_{31})_3$, 25.7; stearin, $C_3H_5(COOC_{17}H_{35})_3$, 1.8; olein, $C_3H_5(COOC_{17}H_{33})_3$, 35.0. If by "fat" we mean "lipids," then small quantities of cholesterol, lecithin, etc., are also present.

The Proteins in Milk.—The known proteins in milk are casein, lactalbumin, and lactoglobulin, the casein constituting 80 per cent of the total protein. Casein is rich in "essential" amino acids; and the other two proteins are also believed to be rich in these substances. Casein is the protein constituent in a synthetic diet for rats which has been used successfully by many investigators in many lands.

*Casein** is a phosphoprotein: it yields phosphoric acid and amino acids on hydrolysis. The isoelectric point of casein is at a *p*H of about 4.6, but the *p*H of milk itself is in the neighborhood of 7.0. This means that casein in milk is in alkaline combination, probably in the form of calcium caseinate.

Casein itself is insoluble in water. The addition of acid to milk precipitates this protein. The same process is accomplished by allowing the milk to stand for some time, when the lactic acid bacteria convert the lactose to lactic acid, which, in turn, precipitates the protein. The casein precipitated under these conditions can be redissolved in alkali and reprecipitated by acids. This is, in fact, one method of purifying the protein.

Milk forms a clot upon the addition of rennin (the rennet of commerce). The composition of this clot has been the subject of much speculation. It is believed that the rennin first changes the casein to another compound, paracasein. Some are of the opinion that this change involves the splitting of one molecule of casein into two molecules of paracasein. At any rate, this changed casein, this paracasein, it is believed, forms the "clot" by combining with calcium. The calcium paracaseinate, unlike the calcium caseinate, is insoluble in water and insoluble in dilute acids and alkalis. It is quite different from the precipitate obtained by the addition of acid to milk or by the addition of ammonium sulfate to milk. One thing is certain: the clotting of milk does not take place in the absence of calcium.†

* Casein is probably a mixture of two proteins, α- and β-casein

† Compare the action of calcium in the clotting of milk with the action of the same element in blood coagulation (Chap 13).

Casein, unlike the lactalbumin and the lactoglobulin, does not coagulate on heating. The skin which forms when milk is boiled is vaguely referred to as a mixture of the protein with the fat.*

Lactose.—The carbohydrate in milk is lactose or milk sugar. It is a rich source of energy, similar to cane sugar, and is often of value in aiding lactic acid bacteria, thereby lessening the amount of undesirable putrefactive bacteria in the intestine.

The Inorganic Constituents of Milk.—The inorganic constituents are many, but only a few are present in any quantity. Among the latter we can include calcium, phosphorus, potassium, sodium, chlorine, and magnesium. Iron is present but in small amounts. The demands for this element by the organism are such that additional iron must be obtained from other foods. Spectroscopic studies have revealed traces of many elements. Copper, for example, is present to the extent of perhaps 0.15 mg. per liter of milk; and yet this trace in conjunction with iron is of importance in preventing an anemia (Chap. 13). The readiness with which "traces" and "impurities" have been dismissed as of no importance is giving place to a more cautious and critical attitude.

Human versus Cow's Milk.—Human milk contains less protein (1.2 per cent) and more sugar (6.5 per cent) than cow's milk. Even when cow's milk is properly diluted and the correct amount of lactose added, the "artificial human milk" is not always a complete substitute for the natural milk when feeding the young. Whatever differences there are are obscure.

Certified milk is raw milk of higher purity. It must not contain more than 10,000 bacteria per cubic centimeter of milk and must not be more than thirty-six hours old when delivered.

Grade A raw milk has an average bacterial count not exceeding 50,000 bacteria per cubic centimeter at the time of delivery to the customer.

Pasteurized milk is "milk that has been subjected to a temperature not lower than 145° F. for not less than thirty minutes."

The composition of milk (from several sources) and of milk products is given in Tables 16, 17 and 18.

Cream usually contains about 20 per cent of fat, although in the "heavy" cream fat may reach as high as 40 per cent.

Butter, as defined by Federal food laws, is "the clean sound product, made by gathering in any manner the fat of fresh or ripened milk or cream into a mass, which also contains a small portion of other milk constituents, with or without salt, and contains not less than 80 per cent of milk fat."

In the manufacture of butter, cream is usually pasteurized and then ripened. The ripening—an acid fermentation—is accomplished by inoculating with the desired organisms. The next process, that of churning, separates the fat globules, which can be drawn off and thereby separated from the butter milk.

* Industrially, casein in solution with certain compounds develops adhesive qualities, and is used in glues, coatings, and the like. With other combinations, it is used in plastics and paints.

Margarine, or *oleomargarine* as it is sometimes called, is coming into use more and more as a butter substitute. It must contain at least 80 per cent of fat by weight. Its fat content is made up of variable mixtures of vegetable and animal fats, the basis usually being cotton-

TABLE 16.—AVERAGE COMPOSITION OF MILK OF VARIOUS KINDS

Kind of milk	Water.	Protein (N × 6.37).	Fat.	Lactose (by difference).	Mineral matter (ash).	Fuel value per pound
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Calories*
Human	87.5	1 4	3 7	7 2	0.2	307
Cow	87 1	3 4	3.9	4.9	.7	310
Goat	87 0	3.3	4.2	4.8	.7	318
Sheep	82 6	5 5	6.5	4.5	.9	447
Reindeer	63.7	10.3	19.7	4.8	1 5	1078

* Compiled by Food Composition Section, Bureau of Home Economics.

TABLE 17.—AVERAGE COMPOSITION OF MILK AND MILK PRODUCTS*

Product.	Water.	Protein (N × 6.37).	Fat.	Lactose,† etc. (by difference).	Mineral matter (ash).	Fuel value per pound.
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Calories*
Whole milk	87.1	3.4	3.9	4.9	0.7	310
Cream:						
Single	72 5	2 9	20 0	4.0	.6	942
Double	54 4	2 2	40 0	3.0	.4	1727
Skim milk	90.5	3 5	.2	5.0	.8	162
Buttermilk	90.7	3 5	.5	4.6	.7	167
Whey	93.0	1.0	.3	5.1	.6	123
Evaporated milk, unsweetened	73.7	7.0	7.9	9.9	1.5	629
Condensed milk, sweetened	27 0	8.1	8.4	‡54.8	1.7	1484
Dried whole milk	3.5	25 8	26.7	38.0	6 0	2248
Dried skim milk	3.5	35.6	1.0	52.0	7.9	1630
Butter	15.5	.6	81.0	.4	2.5	3325
Cheese:						
American cheddar	34.5	25.6	34.7	1 9	3 3	1916
Swiss	34.0	28.6	31.3	1.9	4.2	1831
Cottage (skim milk)	74.0	19.2	.8	4.3	1.7	459
Cream	42.7	14.5	39.9	1 0	1.9	1910

* Compiled by Food Composition Section, Bureau of Home Economics.

† Including lactic acid and other undetermined substances. The amount of sugar in some of the cheeses is probably negligible.

‡ Mainly added sucrose. Average percentage of added sugar is about 42 per cent of the condensed milk.

seed and soybean oils, mixed with smaller quantities of lard and other animal fats. An agreeable flavor is given to the product by churning these fats and oils in specially cultured skim milk, and the nutritional value is enhanced by incorporating vitamin A.

"When margarine is fortified with vitamin A . . . it can be sub-

TABLE 18.—NUTRIENT VALUE OF DIFFERENT FORMS OF MILK

[Bowes and Church, *Food Values of Portions Commonly Used* (Philadelphia Child Health Society)]

Type of milk.	Quantity.	Energy (calories).	Protein (grams).	Carbohydrate (grams).	Fat (grams)	Calcium (grams)	Phosphorus (grams).	Iron (grams)
Milk, whole . . .	6 oz. (1 medium glass)	123	6.1	8 8	7 0	0 212	0 167	0 40
Milk, condensed. .	1 tbsp., or 15 grams (to make 1 glass)	49	1 2	8 2	1 3	0 045	0 035	0 10
Milk, buttermilk	6 oz. (1 medium glass)	65	6 3	9 0	0 4	0 189	0 175	0 50
Milk, evaporated . ..	1 tbsp., or 16 grams (to make 1 glass)	21	1 1	1 5	1 2	0 040	0 032	0 03
Milk, malted. ...	1 tbsp., or 9 grams (to make 1 glass)	38	1 3	6 4	0 8	0 032	0 031	0 20
Milk powder, skim .	28.35 grams (1 ounce)	102	10 1	14 7	0 3	0 346	0 272	0 90
Milk powder, whole	28.35 grams (1 ounce)	141	7 3	10 8	7 6	0 261	0 201	0 40
Milk, skim	6 oz. (1 medium glass)	65	6 3	9 0	0 4	0 220	0 173	0 50

stituted for butter in the ordinary diet without any nutritional disadvantage" (Council on Food and Nutrition, A.M.A.).*

Skim milk is milk from which most of the fat has been removed; which means that its fuel value is low, and that its content of vitamin A is very small. From the standpoint of the manufacturer, skim milk is the most important by-product of milk. Casein, skim-milk powder, and condensed, cultured, and chocolate milks are prepared from it. A large amount is fed to farm animals.

Buttermilk is comparable to skim milk in composition. It is the product obtained after the removal of the fat of the milk during the course of butter-making.

Cheese is essentially a concentrate of the casein and fat of milk. The manufacture of cheese is usually based on the coagulation process resulting from the action of rennin on casein The variations in cheese depend upon the bacteria, the source of milk, the extent of ripening, the temperature used, etc.

After the cheese has been removed, what is left is known as "whey." The important constituent in whey is lactose. From an industrial point of view, this lactose is valuable because it can be converted to lactic acid by a fermentation process.

Homogenized milk is produced by forcing milk through minute openings under high pressure. The fat becomes more evenly distributed, and the fat globules are smaller than in milk.

Condensed milk is milk from which a certain amount of water has been removed and to which some sugar has been added. *Evaporated milk* is milk with less than its usual water content, but here no sugar has been added.

In the preparation of condensed milk, some 18 pounds of sugar are used for 100 pounds of milk, and the mixture is evaporated *in vacuo* at a temperature close to that of pasteurization. In the evaporated, or unsweetened, milk, much of the water from fresh milk is removed (*in vacuo*); the fat globules are broken up into smaller sizes by means of a "homogenizer"; and the product, run into cans and sealed, is sterilized.

Dried milk is milk from which practically all the water has been removed. From 100 pounds of milk some 13 pounds of the white powder may be obtained.

In war time, with large food exports from the United States to England and elsewhere, the saving of shipping space by using the dried foodstuffs was of the utmost importance.

Dried skim milk contains practically none of the fat of whole milk.

Fermented milk, buttermilk and acidophilus milk for example, is a milk which has been acted upon by desirable bacteria, of which the lactic acid bacteria are abundant. Buttermilk is the product left in the churn after the butter has been removed. The cream is usually churned when sour, and the buttermilk is therefore slightly acid.

* Mineral oil (liquid petrolatum), sometimes used in the place of other oils and fats, is chemically not a fat at all but largely a mixture of hydrocarbons. It is objectionable in foods because it interferes with the absorption of a number of the vitamins, such as A, D and K.

Milk Fortified with Vitamin D.—Few common foods contain vitamin D in any quantity. This is the reason why milk is at times enriched with the vitamin The addition of other vitamins, or the addition of various minerals, is hardly necessary for an individual whose diet is varied.

Ice cream consists of cream or milk fat, sugar, flavoring matter, and binder (such as gelatin). The composition varies widely. The milk fat may range from 10 to 14 per cent, and the total solids, including protein and carbohydrate, from 20 to 30 per cent.

Eggs are valuable in nutrition mainly for their content of protein and lipids. The protein, high in the biological scale, is found largely in egg white (ovalbumin and ovoglobulin) and also in the yolk (ovovitellin, a phosphoprotein). The lipids (fat, lecithin, cholesterol) are largely in the yolk. An approximate composition of the egg as a whole is (in per cent): protein 13.4, fat 10.5, ash 1.0, water 73.7. The egg is rich in a number of vitamins.

Meat, according to Federal authorities, "is the properly dressed flesh derived from cattle, from swine, from sheep, or from goats . . . "; and flesh is "any clean, sound, edible part of the striated muscle of an animal." Like eggs, meat is of particular value for its content of protein and fat; and like eggs again, it has very little carbohydrate. Lean meat may contain (in per cent): protein 15 to 20, fat 8 to 14, ash 1, and water 65 to 75. The proteins are myogen, a water-soluble substance, and myosin, a globulin and water-insoluble. A small quantity of glycogen (usually less than 1 per cent) is also found in muscle substance. "Extractives," which give rise to the flavor of meat, include creatine (Chap. 18) and such purine bodies as xanthine and hypoxanthine (Chap. 5). The mineral salts are characterized by their relatively high percentage of potassium and phosphorus.

Fish as a food is, to a considerable extent, similar to meat or to eggs. In fact, these three foods can largely replace one another in the diet. An approximate composition of fish (in per cent) is: protein 10.9, fat 2.4, ash 0.7 (including, in the case of marine fish and shellfish, appreciable quantities of iodine), water 44.6, refuse 41.6. The fat of mackerel may be as high as 16 per cent. Vitamins are also present.

Cereals are the edible portions (grains) of the grass family, *Gramineae.* The grain, according to Federal authorities, is "the fully matured, clean, sound, air-dry seed of wheat, maize, rice, oats, rye, buckwheat, barley, sorghum, millet or spelt." For Americans, wheat is the most important cereal, being used very largely in the form of bread. Twenty-five per cent of the average caloric intake in the United States is due to this cereal. Some of the other cereals are used for breakfast foods.

An approximate composition of wheat (in per cent) is: protein 11.9, fat 2.1, carbohydrate 71.9, fiber (cellulose, etc.) 1.8, ash 1.8, moisture 10.5. The two principal proteins are gliadin (a prolamin, p. 44) and glutenin (a glutelin), with smaller quantities of edestin (a globulin, p. 44). The "gluten" of flour consists very largely of gliadin and glutenin.

Most wheat flour milled in this country is converted into bread

which is made from a mixture of flour and water, fermented with the production of carbon dioxide by an appropriate "leavening" agent, and subsequently baked. The nutritive value of bread depends upon the flour used in preparing it. The milling process has little effect on the protein and carbohydrate, but the fat and ash are reduced in quantity. In the manufacture of white flour, with the conse-

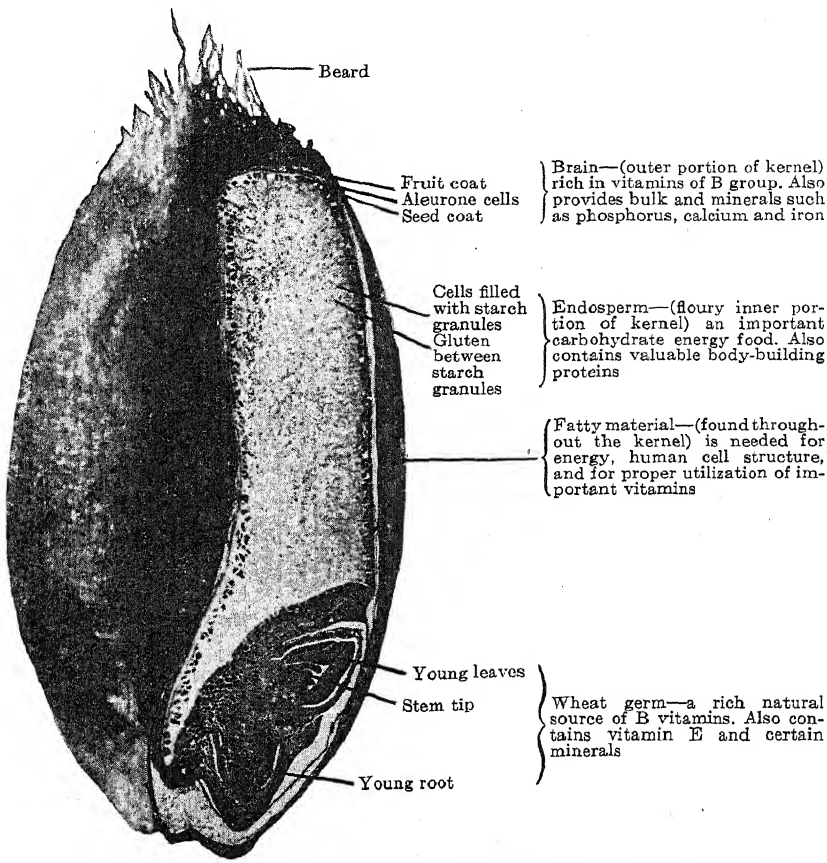

Fig. 35.—The wheat kernel (with longitudinal section exposed). (General Mills, Inc.)

quent removal of the bran and germ, one-half of the calcium is lost, and there are definite losses in phosphorus and iron.

Whole wheat flour is milled to contain 100 per cent of the wheat kernel. White flour contains that part of the wheat kernel called the "endosperm" (Fig. 35). "Enriched" flour is white flour with various additions such as thiamine (p. 148), nicotinic acid (p. 159), iron, etc.

Whole wheat is a very good source of iron, but some four-fifths is lost when white flour is produced. The thiamine (p. 148), riboflavin (p. 153) and nicotinic acid (p. 159) present in the whole wheat are also largely lost in the milling process to produce white flour.

If "enriched" bread is desired, one of several methods may be

adopted. "Enriched" flour may be used. Or the dough may be mixed with milling products of wheat or with a special preparation of some of the missing elements. Still another method is to employ an "enriched" yeast.

In England, during World War II, a flour of 85 per cent extraction of the grain was used in the place of the normal 70 per cent. An 85 per cent extraction means that 15 per cent of the whole grain is not incorporated in the flour. The bread is further enriched with calcium salts—an addition which is sometimes, but not always, made to "enriched" bread in this country.

Wheat flour contains practically none of the vitamins C and D (see next chapter), and very little A, though it does contain an appreciable amount of the water-soluble B vitamins.

In the United States the various cereals contribute about one-third of the calories to the diet; in Europe they very often contribute up to 50 per cent.

Sugar is practically pure carbohydrate and, therefore, rich in energy-yielding material. Before the period of war rationing, of the seven million tons used per year, 60 per cent was used by households and restaurants. Other uses were in industries connected with baking, canning, flavoring, soft drinks, dairying, tobacco, etc.

The common food sugars include cane sugar or sucrose, milk sugar or lactose, malt sugar or maltose, glucose or dextrose, and fructose or levulose.

The cane sugar is derived from the sugar cane and the sugar beet. Malt sugar is made from the partial digestion of starch. The "malt food" for infants contains maltose and dextrin. Glucose is obtained from starch but occurs also as such in nature. The "corn syrup" of commerce is a mixture of glucose and dextrin. Fructose is found in honey—which also contains glucose—and in many fruits.

Maple sugar and maple syrup represent the sap of the sugar maple. The mother liquor left after the removal of part of the cane sugar from the boiled juice is "molasses."

On the whole, much too much sugar (in various forms) is used in this country. The quantities which are consumed lessen the desire for other—and more important—foods.

Fruits and vegetables contribute relatively little as sources of energy, but they are nevertheless very important for their content of minerals and vitamins. Leafy, green and yellow vegetables include asparagus, yellow sweet corn, beets, lettuce, parsley, spinach, water cress, etc. They are rich in vitamin A (p. 138) and, as a rule, in iron. Citrus fruits (orange, lemon, grapefruit, etc.) and tomatoes are splendid sources of vitamin C (p. 173). The presence of cellulose lends bulk to the food and aids in proper digestion. The average composition of a vegetable such as cabbage in (in per cent): protein 1.4, fat 0.2, carbohydrate (including fiber) 5.6, ash 1.8, water 77.7, refuse 15.0. The average composition of a number of fruits is (in per cent) protein 1.0, fat 0.7, carbohydrate (including fiber) 13.0, ash 0.6, water 63.0, waste 22.0.

The potato is a food of high energy value, rich in carbohydrates, relatively poor in protein, and relatively poor in minerals and vitamins. It is a cheap source of iron and vitamin C.*

Cooking.—To a greater or less degree, cooking affects more particularly the vitamins and minerals of foods. So variable are the various factors—amount of water, length of cooking, temperature, etc.—that no one statement will cover all the possibilities.†

Processing of Foods.—Various methods for preserving foods have been developed. These methods received a great impetus during World War II, when urgent demands arose not only for foods which did not deteriorate with time, but which occupied considerably less space than the untreated food (which contains varying quantities of water). The processes employed come under the following headings: drying or dehydration; sterilization and canning; low temperature chilling and freezing; pickling, smoking, spicing and fermentation; etc. The products obtained by these means are of the utmost value when fresh foods are unattainable.

There is, of course, always the danger that foods so prepared have lost some of their nutritional value. This applies more specifically to some minerals and to a number of vitamins of varying stability, such as carotene, vitamin A, thiamine, riboflavin and ascorbic acid. The extent to which such foods are undamaged depends upon a number of factors: exposure to light; temperature and length of time of heating and of standing; the *p*H of the solution; the extent of loss of water-soluble material; etc.

These and other factors are being rigorously investigated in the hope that processed foods may be prepared which, from the nutritional point of view, compare favorably with the unprocessed variety.

In the meantime, canned fruits and vegetables are now prepared with little loss of ascorbic acid—a notoriously labile vitamin.

It would, of course, be to the advantage of the consumer if com-

* Coffee, tea, chocolate, flavoring spices, add to the "spice of life" but are not in themselves necessary foods.

† The following, taken from the *Manual of Industrial Nutrition* (Government Printing Office, Washington, 1943), are several practical illustrations of things to bear in mind:

Use fresh vegetables and fruits as soon as possible after delivery. Handle very carefully for bruising causes rapid losses of vitamins Keep vegetables and fruits crisp and cool until time to cook them. Shred or chop vegetables and fruits just before they are to be served or cooked.

Add vegetables or fruits to rapidly boiling water. Cook quickly and in as little water as possible. Do not add soda to vegetables or fruits to preserve their color because it destroys the vitamins. Cook until just done with some of the original crispness left. Do not stir or expose to air and light any more than absolutely necessary. Do not let vegetables or fruits stand in water. Standing destroys vitamins. Use vegetable cooking water in gravies, soups or sauces. Bring precooked canned fruits and vegetables quickly to a boil but do not continue boiling. Add frozen vegetables and fruits directly to boiling water. Do not defrost preliminary to cooking.

With meats, short methods of cooking such as sauteing or broiling are less destructive of vitamins than slower methods. Roasting at a low temperature is less destructive than at a high temperature. As with other foods, meats should be served as soon as possible after cooking. Standing in a warmer or on a steam table is accompanied by vitamin losses.

plete analyses—including vitamins and minerals—would appear on the labels of cans.

An adequate diet should include the equivalent of some 3000 Calories (for the adult), about 70 gm. of protein, of which 50 gm. should be of animal origin, from 80 to 90 gm. of fat and from 400 to 500 gm. of carbohydrate. Meat, milk, eggs, and fish supply "first-class proteins," that is, proteins rich in essential amino acids; cereals supply carbohydrates and proteins which are usually not "first-class"; and fruits and vegetables supply minerals and "roughage" (cellulose, etc.). A mixture of such foods contains the more important vitamins. To take care of possible deficiencies in the diet, even on so mixed a fare, it is advisable to consume from one to two glasses of milk a day (Table 19).

TABLE 19.—SAMPLE DIETARY PATTERNS, PREPARED BY FOOD AND NUTRITION BOARD, NATIONAL RESEARCH COUNCIL, TO SHOW WAYS IN WHICH ALLOWANCES MAY BE MET.

	LIST I
Milk	1 pint
Egg	1 daily, if possible. (On days not used, beans, peanuts, cheese, or more milk or meat to be used instead)
Meat, fish or fowl	1 or more servings
Potato	1 or more
Vegetables	2 or more servings. One green or yellow
Fruits	2 or more. One citrus fruit or tomato or other good source of vitamin C
Cereals and bread	Whole-grain or enriched

Other foods as needed to complete the meals.

This list is based on the needs of the average adult.

	LIST II
Turnip greens	1 cup
Sweet potatoes	3
Peanuts	20 nuts or 2 tablespoons of peanut butter
Beans or cowpeas	1½ oz.
Tomatoes	1 cup
Corn meal	3 oz.
Enriched flour	3 to 4 oz.
Milk (fresh, evaporated or dried)	⅓ qt.
Lean pork	Small serving 3 to 4 times a week

Molasses, fat, etc., to complete the meals.

The matter of inorganic salts and vitamins will be taken up later (see Chapters 21 and 8).

Social Problems.—A report by the National Research Council states that in a nation-wide canvass in February, 1943, in which one-day diet records were obtained from selective samplings of the population in each of the forty-eight states, the following percentages of persons had none of the respective protective foods: citrus fruits, tomatoes, or salad greens, 45; dairy products, 34; leafy and yellow vegetables, 25; other vegetables or fruit, 8; meat, fish or poultry, 12; whole grain or enriched products, 3.

In a carefully controlled study entitled, "Food, Health, and Income," by J. B. Orr, the author points out that the consumption of milk, eggs, fruit, vegetables, meat, and fish rises with income. An examination of the composition of the diets shows that the degree of

adequacy for health increases as income rises As income increases disease and death rate decrease, children grow more quickly, adult stature is greater and general health and physique improve. Among the poorer children, the improvement of the diet is accompanied by improvement in health and increased rate of growth. This improvement in diet means increased consumption of milk, eggs, butter, fruits, vegetables, and meat to the extent of from 12 to 25 per cent

A study carried out in Pennsylvania between 1935 and 1940 among children from various communities substantially confirms the general view that as income increases health increases. The family income of the "college community" was definitely higher than that of the "industrial community."

Where family income is limited, the possibilities of varying the diet in sufficient amounts—so important in the attainment of a "wholesome" diet—become more restricted. The poor spend more on flour and cereals, potatoes and sugar, and less on butter and fats, meat, eggs, milk and fruits and vegetables than do the more prosperous portions of the community.

In a study by Jolliffe, McLester and Sherman, these eminent authorities come to the conclusion that dietary inadequacies and malnutrition are of frequent occurrence in the United States. They define "dietary inadequacy" as the failure to ingest an essential nutritional factor or factors in amounts sufficient to meet the existing requirement of the body; and "malnutrition" as a bodily condition, detectable by any method of examination, caused by a nutritional inadequacy.

Another study on the influence of prenatal diet on the mother and child, carried out by Ebbs, Tisdall and Scott of Toronto, and involving 400 women with low incomes, again emphasizes the close relationship between income, wholesome food and good health. In carrying out this study, one group found to be on a poor diet was left as a control, a second group on a poor diet was improved by supplying food during the last three or four months of pregnancy, and a third group, found to have moderately good prenatal diets was improved by education alone.

During the whole course of pregnancy the mothers on a good or supplemented diet enjoyed better health, had fewer complications and proved to be better obstetrical risks than those left on poor prenatal diets. The incidence of miscarriages, stillbirths and premature births in the woman on poor diets was much increased. The incidence of illness in the babies up to the age of six months and the number of deaths resulting from these illnesses were many times greater in the poor diet group.

With the income distribution and food price levels such as existed in 1935–36, it has been estimated that at any one period about one-fourth of the families of the United States would be found to have good—biologically sound—diets, more than a third fair diets, and another third or more poor diets (Stiebeling and Leverton).

These authors point out that if the average consumption of foods could be raised to the level of a "good" diet, the increase (in per cent)

in national consumption of certain basic foods would be as follows: milk 20; butter 15; eggs 35; tomatoes, citrus fruit 70; leafy, green and yellow vegetables 100.

The League of Nations Statistical Year Book for 1938–1939 points out that in New Zealand people live, on the average, to the age of 65; in the United States, to 62; in the United Kingdom, to 60. But the drop is marked when we come to Japan (45), Egypt (30), and India (27.5). There is no doubt in the minds of medical men and nutritional experts that to some extent at least these differences are due to the wide variations in the amount and the kind of food consumed.

To eat adequately—that is, to have a well-balanced diet—means that one must have a certain sum of money to spend and that one must be familiar with the elements of nutritional science. Assuming both these points, we may quote from an excellent pamphlet by Carpenter and Stiebeling, "Diets to Fit the Family Income."

"A liberal diet, as its name implies, provides very generously for all of the food requirements. It contains an abundance of fruits and vegetables, eggs, and lean meat, as well as a generous allowance of milk, along with moderate quantities of cereals, fats, and sugars. This combination of foods allows for better-than-average nutrition, because it provides more than amply for the items necessary for growth, health, and general well-being. At the same time, it offers an assortment pleasing to the eye and the palate, and allows for a great deal of variety from meal to meal."

Table 20 gives the nutritive value of a moderate-cost adequate diet.

It is interesting to compare this table with the one devised by a committee of the National Research Council. This committee first formulated a series of recommendations which is summarized on p. 111. Compare these tables with Table 21, which deals with the approximate food value of the daily allowance of a man moderately active and weighing 70 kilograms. In Table 21 the foods are listed, the approximate measures given, and also the calorific equivalent of each food is listed.

Food in Europe during the War.—In England during World War II this knowledge of food and its functions has been applied with such wisdom that the health of its inhabitants, particularly its children, has shown striking improvement. In the first place the Ministry of Food became the sole purchaser of 90 per cent of the country's food import—and England imports a substantial part of her food supply. In the second place the Ministry of Food insisted that only those foodstuffs which have high nutritive value and occupy relatively little space be imported.

Orange juice was imported in concentrated form. Much of the milk and eggs and some of the meat were shipped in dried form. (Dried eggs, for example, occupy one-sixth the space of the fresh eggs and need no refrigerating plants.)

By means of a rigid system of rationing, coupled with a sound educational program, rich and poor alike received essentials. The chil-

TABLE 20.—NUTRITIVE VALUE OF A MODERATE-COST DIET. [*Family Nutrition.* (Philadelphia Child Health Society.)]

Persons.	Food energy.	Protein.	Calcium.	Phosphorus.	Iron.	Vitamin A	Thiamin.	Riboflavin.	Ascorbic acid.
	Calories.	Grams.	Grams.	Grams.	Milligrams.	International units.	Milligrams.	Milligrams.	Milligrams.
Children:									
9–12 months	970	38	1.1	1.0	6	4,400	0 8	1 9	60
1–3 years	1,300	55	1 3	1 3	7	5,700	1 1	2 4	65
4–6 years	1,710	65	1 3	1.4	9	6,200	1 4	2 6	80
7–9 years	2,130	79	1 4	1 6	12	8,000	1 7	3 0	100
10–12 years	2,670	91	1.4	1.8	14	9,200	2 1	3 3	115
Girls:									
13–15 years	2,980	102	1 5	1 9	16	9,700	2 4	3 5	115
16–20 years	2,590	94	1 4	1 8	15	9,800	2 1	3 3	125
Women:									
Moderately active	2,590	86	1.1	1 6	14	9,700	2 1	2 8	120
Very active	3,250	104	1 2	1.8	17	8,800	2 3	3.1	120
Sedentary	2,260	80	1 1	1 5	13	8,800	1 9	2 7	110
Pregnant	2,750	95	1 5	1 8	15	10,500	2 1	3 4	130
Nursing	3,240	114	2 1	2 3	17	12,100	2 6	4 4	170
Boys:									
13–15 years	3,420	112	1.5	2.0	18	9,600	2 6	3 7	130
16–20 years	4,020	122	1 5	2 2	20	10,200	3 0	4 0	140
Men:									
Moderately active	3,250	104	1 2	1 8	17	8,800	2 3	3 1	120
Very active	4,860	139	1 3	2 3	24	10,500	3 5	4 0	140
Sedentary	2,580	85	1 1	1 6	14	9,000	2 0	2 8	115

TABLE 21.—APPROXIMATE FOOD VALUE OF DAILY ALLOWANCE OF MAN, MODERATELY ACTIVE, AND WEIGHING 70 KG. [*Proc. National Nutrition Conference for Defense.* (Washington, 1941.)]

Foods.	Amount.	Approximate measure.	Calories.	Protein	Calcium.	Iron.	Vitamin A.	Thiamin (B_1).	Ribo-flavin	Ascorbic acid.
	Grams.			Grams.	Grams.	Mg.	I.U.	Gamma.*	Gamma.*	Mg
Milk	480	1 pint	336	15 8	0 58	0 15	528	244	1,000	6
Meat	100	¼ pound	150	21.0	01	3.00	50	120	225	
Potatoes	350	3 medium	300	7.2	05	3 66	144	432	162	12
Baked beans	200	1 cup	200	13 2	09	4 00	110	235	130	
Cabbage, raw	100	1 cup	25	1.1	04	43	88	70	72	35
Carrots	100	½ cup	40	1 2	04	64	2,100	60	58	
Tomato	200	⅝ cup	50	2 4	.02	.80	2,000	182	122	48
Prunes, stewed	200	⅝ cup	250	1.4	.03	1 88	990	120	132	
Oleomargarine	66	5 tablespoons	500				2,600			
Oatmeal, cooked	300	1¼ cups	200	8.0	.03	2 40	...	270	60	
Bread, whole wheat or "enriched"	200	6 slices	500	19.0	10	3.0	...	480	207	
Gingerbread	75	Large piece	200	3 5	.08	2.0	...	40	30	
Sugar, jam	...	...	250	..						
Totals	...	...	3,001	93.8	1.07	22.0	8,602	2,253* 2.25 mg.	2,234* 2 23 mg.	101
Compared with recommended allowances	...	... ...	3,000	70.0	.80	12 0	5,000	1.80 mg.	2.70 mg.	75

* 1 milligram (mg.) equals 1,000 micrograms (gamma).

dren were particularly favored. Extra milk and noonday meals (carefully selected) became not only common but practically compulsory for all schoolchildren; and those below school age were especially cared for, children under five had priority rights over milk, oranges, eggs, fruit juices and cod liver oil. Expectant mothers and children under five received a pint of milk a day free or at a reduced price.

One other fact of importance should be noted. The "white bread" of the English, deficient in minerals and vitamin B, gave place to "enriched" white bread.

In general, the prewar diet of "tea and margarine" and "white bread" of the poor (and to some extent of the rich) has given place to more butter, milk, eggs, fruits and vegetables.

In France, in Belgium, in Holland during the German occupation, the situation grew steadily worse. In 1943 in Paris, for example, the inhabitants were getting *the equivalent* of about one-half of their normal calorific needs (2,400 to 2,600). Instead of 80 gm. of fats, adults were given 15, and children between three and six, 39.

In Poland, and Eastern Europe, in general, the picture was far worse; thousands died of plain starvation.

Incomplete accounts from Germany during the War suggested that the Germans were much better fed than the inhabitants of the occupied countries.

Nutrition and National Defense.—The importance of adequate nutrition for the nation was vividly brought home during the depression of the 1930's. We must thank the many nutritional authorities in this country for their splendid work in calling attention to the importance of the problem. During 1930–1940 the Federal Government made possible the distribution of dairy products, green vegetables and other protective foods among low-income families. Between 1935 and 1939 the Federal Surplus Commodities Corporation gave to welfare agencies three billion pounds of surplus foods which were distributed to families receiving public assistance.

The acute world situation led President Roosevelt in May, 1941, to call a National Nutritional Conference for Defense, to discuss the problems of nutrition and to formulate recommendations for a national program of action. This body is now acting toward problems of nutrition in much the same way that the general army headquarters acts toward problems connected with the army (Table 21).

A further detailed study of the world food situation was made by the United Nations Food Conference, held at Hot Springs, Va., in May, 1943. The basic problem was stated by Under-Secretary of Agriculture Appleby: Long after the industrial revolution, two-thirds of the people of the world are normally engaged in producing food, and two-thirds of the people of the world do not have enough to eat.

Fortunately for us, the natural wealth of the country, coupled with increased employment and increasing wages, due to war conditions, make the immediate outlook (in so far as adequate food is concerned) encouraging—particularly so when compared with the other nations of the world. One immediate problem of our authorities is to

see that the nutritional knowledge we have becomes part of the necessary information for every man, woman and child in the country.

One result is already apparent. In so far as the American armed forces are concerned, they are splendidly fed. Not only are calorific needs taken into consideration—but the utmost care is taken to ensure biologically wholesome and esthetically attractive meals (see Appendix, p. 567).

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CHAPTER 8

VITAMINS

THAT disease can result from a food deficiency had been vaguely known for many years. Sailors discovered that scurvy could be prevented by incorporating fresh fruit or fresh vegetables in the diet. In 1882 Takaki eliminated beriberi from the Japanese navy by giving increased quantities of meat, barley, and fruit to his sailors. To be sure, he was wrong in his explanation that a sufficient amount of protein prevented beriberi, but he was right in supposing that a food

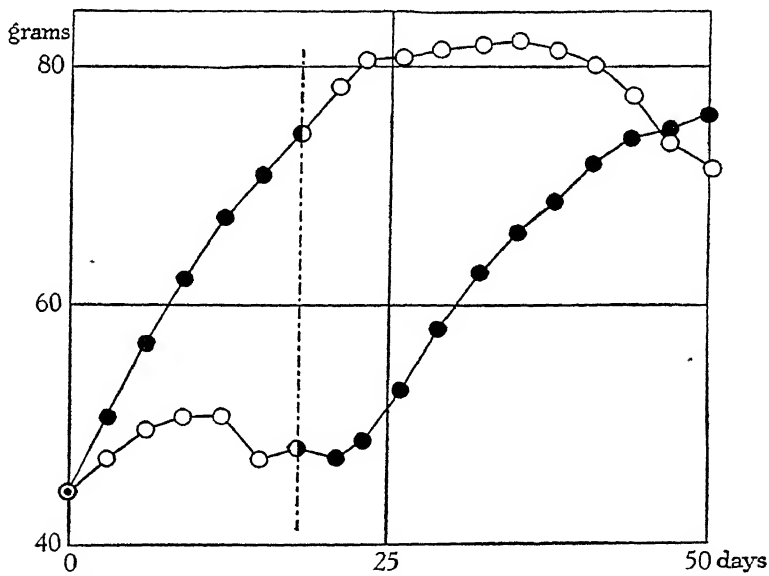


Fig. 36.—Growth curves of rats, with and without vitamins (Hopkins). ○ artificial diet alone. ● Artificial diet plus milk.

deficiency was a causal factor in its development. Toward the close of the nineteenth century, a number of physicians began to recognize the value of cod liver oil in curing rickets.

An impetus for further study was given by Eijkman, a Dutch physician, with his discovery that experimental beriberi could be induced in birds. This occurred in 1897. He found that hens developed the disease when fed with polished rice. Moreover, such hens could be cured by giving them the rice polishings. Still under the influence of Pasteur, Eijkman for a time believed that the rice polishings contained a "something" which neutralized the beriberi "germ" in the polished rice.

An equally important advance we owe to the Norwegians, Holst and Frölich, who, in 1907, caused scurvy in guinea-pigs by feeding them a cereal diet deficient in "greens."

We owe to Funk the first clear evidence of the validity of the vitamin hypothesis. From the very first (1911), Funk regarded the Eijkman factor in beriberi as a definite chemical substance (present in whole rice in relatively small quantities), the absence of which, in polished rice, causes the disease. He boldly attacked the problem of its isolation from rice and from yeast, the latter of which he found to be rich in the antiberiberi factor. He obtained extremely active concentrates, isolating from his fractions nicotinic acid which is only now beginning to assume importance in a study of the vitamin B complex. Incidentally, we owe the name "vitamin" to Funk.

Shortly thereafter (in 1912), Hopkins in England published an important paper on the influence of small quantities of milk when added to a synthetic diet. An artificial diet, consisting presumably of all the important constituents found in milk (protein, fat, carbohydrate, mineral salts, and water), proved deficient; but when 2 cc. of milk was added to the diet of each rat each day, the animals recovered (see Fig. 36). Obviously, there was something in milk other than the hitherto recognized components, which was present in minute quantities and which was essential for the normal development of the animal. This "something" is a vitamin.*

The suspicion that there might be more than one vitamin was strengthened by the work of McCollum and Davis in 1915. They showed that the substitution of lard for egg yolk in a synthetic diet prevented the rats from growing; and that the substitution of highly purified lactose for ordinary lactose prevented growth and also gave rise to polyneuritis. The first factor, associated with fats, was named "fat-soluble A," and the second factor, associated with water-soluble material, was named "water-soluble B." These two substances were later renamed vitamin A and vitamin B, respectively.

The number of vitamins has been rapidly multiplying.

The vitamins are usually divided into those which are fat soluble and those which are water soluble. The fat-soluble vitamins are A, D, E and K; the water-soluble vitamins are the B complex (which includes several vitamins) and C.† For the sake of convenience we take them up in *alphabetical* order.

VITAMIN A

A lack of vitamin A results in loss of weight and decreased resistance in infection. Neither of these symptoms is specific. Wolbach states that characteristic of a deficiency of vitamin A in the animal is the substitution of stratified keratinizing epithelium for normal epithelium in various parts of the respiratory tract, alimentary tract, eyes, and the genito-urinary tract. What is specific is the development of xerophthalmia, a disease in which the eyes become hemorrhagic,

* With a very pure synthetic diet, 2 cc. of milk is insufficient.

† As we shall see presently, there are several varieties of vitamins A, D, E and K.

incrusted, and infected (see Figs. 37 and 38). Probably the earliest symptom is hemeralopia, or partial night blindness (that is, loss of visual acuity in dim light).

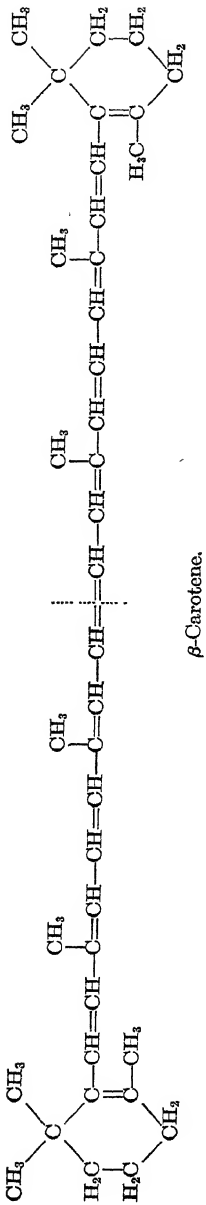
Carotenoids and Vitamin A.—A deficiency in vitamin A can be cured not only by substances containing this vitamin, but by several

Fig. 37.—Xerophthalmia. (*Therapeutic Notes*, Parke, Davis and Co.)

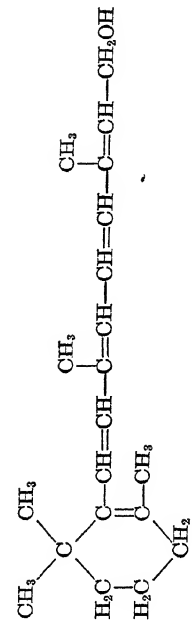
Fig. 38.—Xerophthalmia—an eye trouble caused by deficiency of vitamin A. (C. E. Bloch, from Harris, *Vitamins in Theory and Practice*, Cambridge Univ. Press, Publishers.)

pigments found in the plant kingdom, particularly carotene (provitamin A), which occurs in three isomeric forms, α, β, and γ. The β-carotene gives the most active response. These plant pigments are converted into vitamin A in the animal body.

Karrer and his associates have established the structures of β-carotene and vitamin A.

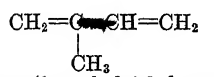


β-Carotene.



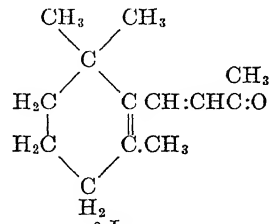
Vitamin A.

The aliphatic central chain of β-carotene represents four dehydrogenated isoprene residues.



Isoprene (2-methyl-1,3,-butadiene).

β-Carotene also contains two β-ionone rings.



β-Ionone.

Vitamin A, an alcohol, represents approximately one-half of the molecule of β-carotene. The vitamin has been isolated in pure form.

There are some 30 carotenoids whose structure is known and several have the β-ionone ring: such as, α-carotene, β-carotene, γ-carotene and cryptoxanthin; these are converted (in varying degrees) into vitamin A in the body. The extent of conversion, however, varies with different persons.

Table 22 gives a list of some of the carotenoids, their sources, and their relation to vitamin A:

TABLE 22.—A PARTIAL LIST OF CAROTENOIDS, THEIR IMPORTANT SOURCES, AND THEIR RELATION TO VITAMIN A.* (Palmer, *J. Am Med Assoc.*, 110, 1748.)

Name.	Sources.	Molecules of vitamin A possible from one molecule of pigment.
α-carotene	Red palm oil, chestnuts, carrot root, mountain ash berries.	1
β-carotene	Green leaves, carrot root, red palm oil, butter.	2
γ-carotene	Fruits of Gonocaryum pyriforme (a Dutch East Indies plant), leaves of lily-of-the-valley.	1
Cryptoxanthin	Red calix and fruit of the Chinese lantern plant, yellow corn, egg yolk, green grass.	1
Xanthophyll (lutein)	Green leaves and grass.	0
Zeaxanthin	Yellow corn, green leaves, egg yolk.	0
Rhodoxanthin	Seed coat of the yew.	0
Astacin	Lobster, salmon, shrimp.	0
Lycopene	Red tomato, watermelon.	0
Capxanthin	Red peppers, paprika, pimiento.	0
Fucoxanthin	Brown algae.	
Taraxanthin	Dandelion, sunflower, cockspur.	0
Violaxanthin	Yellow pansy.	0
Flavoxanthin	Buttercup.	0

* The carotenoids mentioned are not necessarily the exclusive cause of the pigmentation of the products mentioned. In most cases they are not. Of the vitamin A active products, red palm oil and carrots contain chiefly β-carotene and α-carotene and butter chiefly β-carotene, and of the carotenoids in green leaves, β-carotene is found almost exclusively.

(The Council of *the Am. Med. Assoc.* allows the use of the term *Pro-vitamin A* as a synonym for α, β or γ-carotene, or cryptoxanthin, or a combination of two or more of these.)

that bile is necessary as it is, for example, in the case of vitamin K (p. 192). However, since vitamin A, like vitamins D, E and K, is fat-soluble, the requirements for complete absorption of fats (see p. 251) may also apply here.

If there are some doubts as to the manner of absorption of vitamin A itself, there is less doubt about its precursor, carotene; for this substance is less easily absorbed than vitamin A, and bile salts definitely facilitate absorption.

Vitamin A is absorbed through the lymphatics and ultimately enters the blood (compare with fat absorption, p. 251), where it is found in the plasma.

As a rule, little if any vitamin A or carotene is excreted by the kidneys; but carotene, and to a less extent vitamin A, may be excreted with the feces.

While, theoretically, one molecule of β-carotene should yield two molecules of vitamin A (see Table 22), it appears from experiments on rats that scarcely more than one molecule of vitamin A is formed from one molecule of carotene. Somewhat the same picture presents itself in man. The greater difficulty in absorbing carotene as compared to vitamin A may be a determining factor. Vitamin E markedly increases the utilizability of carotene and vitamin A, probably due to its ability to prevent oxidative destruction.

Assay.—Colorimetric methods, depending upon the blue color formed with antimony trichloride (p. 142) are used. However, many careful assays still employ the tedious biological test methods. Here young rats are put on a diet devoid of vitamin A [for example, purified casein, starch, vegetable oil, salt mixture, yeast (vitamin B complex), vitamin D, water], and when they show decline in weight, the tested material is added and compared with a standard sample of the vitamin.

Both these methods can be applied either to vitamin A or to carotene.

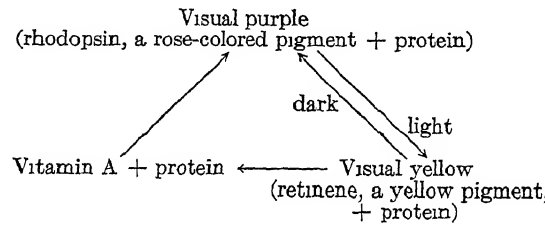
A method for vitamin A which is frequently used depends upon the measurement of the absorption of ultraviolet light at the maximum (328 mμ) of the vitamin A absorption band. Still another method is to measure the absorption of light at the maximum (620 mμ) of the absorption band of the blue colored reaction product formed when vitamin A and antimony trichloride react. Such methods can also be applied to carotene.

Xerophthalmia (Fig. 38), the eye disease so characteristic of a deficiency of vitamin A, is rare in this country and in Europe, although during World War I (1914–1918) several cases came to the attention of physicians. One such notable example occurred in Denmark in 1917.

Night Blindness.—In the earlier stages of the deficiency, there may develop a night blindness (nyctalopia) for which vitamin A is a specific remedy. The researches of Wald and of Hecht have opened up a fertile field in connecting vitamin A with visual purple, a complex pigment (pigment plus protein) present in the eye. In dim light, vision is due to the activity of small elements in the retina known as rods. It is

these rods which contain the visual purple. When light acts on this pigment it is bleached; and this process is the first of a series of processes involved in vision. Where there is a deficiency of vitamin A, visual purple is no longer formed with the normal speed or in normal quantities.

The action of light on visual purple, according to Wald, gives rise to a yellow pigment, retinene, which belongs to the group of carotenoids of which carotene is an example. This retinene is converted by the retina into vitamin A, which is practically colorless. The vitamin A then becomes a source for visual purple. The cycle may be viewed as follows:



In the absence of sufficient vitamin A, less visual purple is formed and night blindness develops.

Dark Adaptation.—"On coming from a brightly illuminated outdoors into a dimly lighted room," writes Hecht, "one can see hardly anything at first, but, as one stays indoors, objects slowly take shape, and after fifteen or twenty minutes they appear so clear that one recalls the initial visual obscurity with astonishment. The process of achieving this good vision is called 'dark adaptation.'"

The phenomenon of "dark adaptation" can be used to measure vitamin A deficiency. This is due to the fact that the rate of regeneration of visual purple depends upon the amount of vitamin A in the body. When there is a deficiency of vitamin A, the process of regeneration is retarded.

Among the early clinical effects of vitamin A deficiency is an impairment of dark adaptation. The biophotometer is one instrument used for measuring such deficiencies.

During World War II some one million men of draft age were estimated to be deficient in color vision. In general, treatment with vitamin A did not result in any marked improvement.

Infection.—Rats on a diet deficient in vitamin A are very prone to infection. Harris believes that this increased susceptibility is connected with the "drying up" of the mucous membrane, which then ceases to secrete mucus. In any case, the administration of vitamin A to infected animals has no influence in mitigating the severity of the disease. In fact, as Moore has shown, animals can die of an infectious disease and still retain appreciable quantities of vitamin A in the liver (where it is stored).

Vitamin A Values of Foods.—Foods derived from the plant kingdom show a correlation between greenness and the amount of vitamin

A or carotene. Green leaves are excellent sources of the pro-vitamin. Such examples are the outer green leaves of lettuce and of cabbage. Other rich sources are green seeds and seed foods (peas, beans, etc.), fleshy vegetables (green peppers, etc.), and green stems (asparagus, broccoli, celery, etc.).

Very often the yellow color of the foodstuff (due to carotene) is a good indication of vitamin A activity. Such examples are carrots, sweet potatoes, apricots, yellow peaches, and bananas.

Cereal grains, nuts, and legumes are, as a rule, poor sources of pro-vitamin A. Yellow corn is a notable exception.

Among foods of animal origin, eggs (excellent) and whole milk and milk products (from good to excellent) are sources of vitamin A.

As has already been pointed out, the livers of the cod, halibut, shark, etc., are rich in this vitamin.

Foods stored in the frozen state retain their content of vitamin A—to a very large extent, at least. Dried and dehydrated foods reveal considerable losses of the vitamin, probably due to the oxidation of the vitamin in the course of drying. Foods which lend themselves to storage for some time in their natural state lose little of their content of the vitamin over a period of from nine to twelve months.

Two Vitamins A.—In the retinas of mammals, frogs, and marine fishes in general, rhodopsin, the rose-colored photosensitive pigment, is changed into vitamin A. In the rods of certain fresh-water fishes Wald has shown that rhodopsin is replaced by a purple photolabile pigment to which he has given the name *porphyropsin*. The absorption bands of the product arising from porphyropsin (with and without antimony trichloride) are different from those obtained with the product (vitamin A) obtained when rhodopsin undergoes its transformation. This has suggested that there are two varieties of vitamin A; and as a matter of convenience the product arising from rhodopsin has been called vitamin A_1, and that arising from porphyropsin, vitamin A_2. Table 23 summarizes the results:

TABLE 23.—ABSORPTION MAXIMA OF SOLUTIONS OF RHODOPSIN AND PORPHYROPSIN IN AQUEOUS DIGITONIN, OF SOLUTIONS OF VITAMINS A AND RETINENES IN CHLOROFORM, AND OF ANTIMONY CHLORIDE REACTIONS WITH THE LATTER SUBSTANCES IN CHLOROFORM. (Wald, *J. Gen. Physiol.*, **22**, 391.)

Substance.	Absorption maximum.	Antimony chloride maximum.
	$m\mu$	$m\mu$
Rhodopsin	500	
Retinene$_1$	387	664
Vitamin A_1	328	615–620
Porphyropsin	522	
Retinene$_2$	405	705
Vitamin A_2	355	696

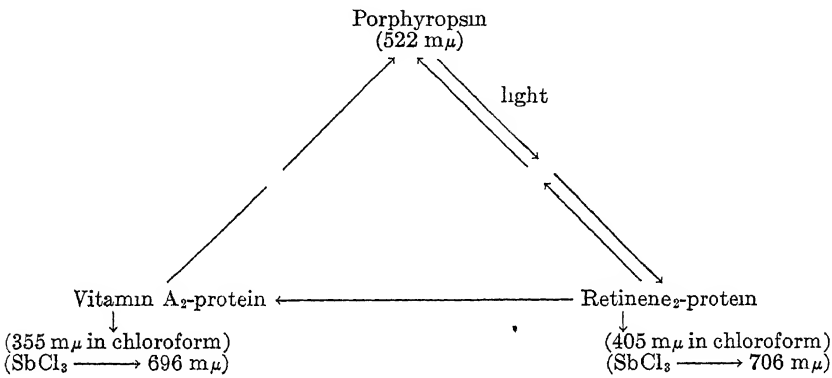
Wald has made the interesting observation that, in general, the eye tissues of permanently marine fishes contain vitamin A_1, while those

of permanently fresh-water fishes contain vitamin A_2. The migratory fishes contain mixtures of both vitamins.

Whether such general conclusions can be applied to the livers of the fish is a question. In at least one case, the northern pike (*Esox lucius*), all the vitamin A stored in the liver is in the form of A_2.

The chromogen responsible for the 696 mμ band (due to vitamin A_2) has also been observed in the liver oils of fresh-water fish.

The porphyropsin cycle may be given in the following diagram (compare with p. 144):



The formula for vitamin A_2 is not known definitely, but it probably resembles closely vitamin A (or A_1).

The Council on Pharmacy and Chemistry* of the American Medical Association summarizes our knowledge of vitamin A as follows:

Evidence for the existence of vitamin A and its role in human nutrition is based on the fact that a characteristic eye disease, usually called xerophthalmia, results from a deficiency of this vitamin.

It is generally agreed that the first symptom or at least one of the first clinical symptoms of vitamin A deficiency is night blindness, or nyctalopia. For this type of night blindness vitamin A is a specific. Cases with nyctalopia exist which do not respond to treatment with vitamin A. These may be due to congenital defects or to other diseases than avitaminosis "A." In view of present knowledge, the claim is not acceptable that the administration of vitamin A to drivers of automobiles will diminish the chance of accident from driving at night.

Vitamin A is reported to be effective in the treatment of certain types of hyperkeratosis [hypertrophy of the corneous or horny layer] of the skin of persons suffering from severe deficiency of vitamin A.

Vitamin A in excess of normal requirements has not been shown to be of value in the prevention of colds, influenza and such infections.

There is at the present time inadequate evidence to warrant the claim that the ingestion of sufficient vitamin A will prevent the formation of renal calculi in man or that it is useful in the treatment of hyperthyroidism, anemia, degenerative conditions of the nervous system, sunburn, or ulcerative conditions of the skin.

The table on p. 111 gives the recommended daily allowances for vitamin A.

It must be pointed out, however, that a number of investigators believe that the figure given—5,000 units—is insufficient. Some advocate an increase to an intake of 7,500 to 10,000 units. The higher figures take into account the incompleteness of absorption.

* New and Nonofficial Remedies (1944).

THE VITAMIN B COMPLEX

Originally, vitamin B referred to the vitamin the absence of which gives rise to beriberi in man and polyneuritis in birds. The work of Goldberger on pellagra led to the view that vitamin B consisted of at least two factors, the heat-labile antiberiberi factor, and the comparatively heat-stable antipellagra factor. Some called the former the true vitamin B, and others, vitamin B_1; some called the heat-stable factor vitamin G, and others vitamin B_2.

In 1933 Kuhn, P. Gyorgy and Wagner-Jauregg isolated what they supposed at the time was vitamin G (B_2) and found it to be a flavin. That this flavin—now known as riboflavin—was not the sole antipellagra factor became apparent when it was shown that the pigment does not cure human pellagra, and that a combination of vitamin B_1 plus riboflavin does not prevent dermatitis in rats. Riboflavin, then, had little, if anything, to do with pellagra; and it became obvious that the so-called vitamin B_2 consisted of more than one factor.

Elvehjem and coworkers next found that after removing the flavin from liver extract (a good source of much of the vitamin B complex) by adsorption on fuller's earth, the residue cured pellagra-like symptoms in chicks and blacktongue in dogs. But Elvehjem's arresting contribution was the discovery that nicotinic acid cures canine blacktongue (the analogue of pellagra in man); and he succeeded in isolating the amide of nicotinic acid from highly active concentrates of liver extract.

But the vitamin B complex, as represented by yeast, rice bran and liver extracts, contains still other factors. These substances have been discovered by showing that the isolated constituents are still not the equivalent in biological response to the yeast or liver or rice bran from which they were extracted.

The substances comprising the vitamin B complex are the following:*

(a) *Thiamine* (vitamin B_1, antineuritic factor, aneurine, heat-labile factor)
(b) *Riboflavin* (vitamin B_2, vitamin G), a growth factor
(c) *Niacin* [nicotinic acid or nicotinic acid amide, P-P factor], pellagra preventive factor
(d) *Pyridoxine* (vitamin B_6), antidermatitis factor
(e) *Pantothenic acid* (filtrate factor), chick antidermatitis factor and necessary for growth of rats.
(f) *Biotin* (vitamin H, coenzyme R, anti-egg white injury factor), needed for the growth of yeasts, molds and bacteria
(g) *Para-aminobenzoic acid*, an anti-gray hair factor (?)
(h) *Inositol* (mouse anti-alopecia† factor), promotes growth in the chick and cures "spectacle eyes" in rats
(i) *Choline*, a growth factor. It also prevents perosis‡ in chicks and is needed for methylating compounds in the body (p. 337)
(j) *Folic acid*, a growth factor for chicks (?) and bacteria

* The list is admittedly still tentative.
† alopecia = baldness.
‡ perosis = a shortening and thickening of the bones.

Thiamine, also called vitamin B_1. A deficiency of thiamine primarily involves the nervous and circulatory system. In the absence of this vitamin a peripheral neuritis sets in, resulting in paralysis. The beriberi of man has its corresponding analogue in the polyneuritis of the bird and the rat (Figs. 39, 40). Not only is there the typical paralysis, but cardiovascular symptoms, edema, and loss of appetite are noticed.

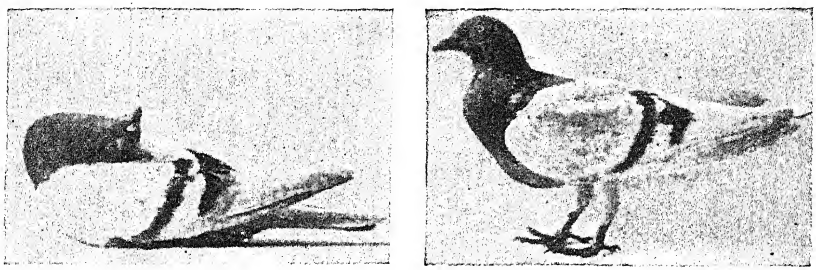


Fig. 39.—Before and after. The effect of thiamine given to a pigeon with beriberi. (Drummond in Plimmer's *Vitamins and Choice of Food,* Longmans, Green & Co.)

Fig. 40.—The white animal exhibits typical convulsive movements due to degenerative changes of the inner ear resulting from thiamine starvation. The position of the hind legs of this animal is typical of the so-called "polyneuritis" of rats, due to lack of vitamin B_1. The black animal exhibits uncoordinated movements due to lack of vitamin B_1, but the condition of thiamine starvation has not developed to the stage of the typical convulsive movements. (I. F. Harris.)

In addition to typical cases of beriberi, comparatively rare in this country, there are several related diseases which are not so uncommon. For example, there are the several polyneuropathies, associated with faulty absorption, restricted food intake and excessive excretion (diarrhea); infectious polyneuritis, alcoholic polyneuritis, polyneuritis of pregnancy, etc. The Wernicke syndrome, characterized by ophthalmoplegia (paralysis of eye muscles), polyneuropathy and clouding of consciousness, is partly caused by a thiamine deficiency.

Function.—It is well known that thiamine plays a role in carbohydrate metabolism. In thiamine deficiency there is interference with glycogen storage, hyperglycemia, etc. Peters and others are of the opinion that this vitamin acts as a coenzyme in facilitating the oxidation of pyruvic acid in the body. This pyruvic acid, $CH_3.CO.COOH$, is an intermediate product of carbohydrate metabolism. In the absence of thiamine, the oxidation of pyruvic acid is impaired. In acting as coenzyme, the vitamin is joined to phosphoric acid (thiamine pyrophosphate or cocarboxylase, p. 540).

Aside from its connection with pyruvic acid, the probably broader role of thiamine in metabolism is not known.

Where there is a definite thiamine deficiency, the amount of pyruvic acid increases in the tissues—a fact made use of in clinical diagnosis.

Distribution.—Table 24 gives the food sources of thiamine.

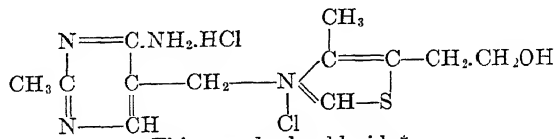
TABLE 24.—FOOD SOURCES OF THIAMINE (VITAMIN B_1). (Daniel, *Food and Life.*)

Type of food.	Excellent sources.	Good sources.	Fair sources.
Animal products.	Lean pork, chicken, kidney, liver.	Egg yolk, brains, lean beef, lean mutton, fish roe, codfish, sardines, whiting.	Fresh milk (whole or skim).
Vegetables .	Green peas, green lima beans.	Potatoes, sweet corn, sweet potatoes, Brussels sprouts, cauliflower, cabbage, mushrooms, spinach, turnip greens, water cress, garden cress, lettuce, collards, kale, onions, leeks, tomatoes, wax and green beans, parsnips, beets, carrots.	Turnips, broccoli, kohlrabi, eggplant.
Fruits ..	.....	Prunes, avocados, pineapples, oranges, grapefruit, tangerines, dates, figs, plums, pears, apples, cantaloups.	Bananas, watermelons, raspberries, blackberries.
Seeds... .	Wheat germ, corn germ, rye germ, rice polishings, wheat bran, oats, whole-grain wheat, rye, barley, brown rice, peanuts, soybeans, cowpeas, navy beans, dried peas.	Hazelnuts, chestnuts, brazil nuts, walnuts, almonds, pecans.	

In foods (and in tissues) the vitamin occurs in the free state and also as the pyrophosphate (cocarboxylase).

Isolation.—The vitamin has not only been isolated in the chemically pure state, but it has also been synthesized. The original isolation we owe to two Dutchmen, Jansen and Donath, who developed an elaborate

series of operations for the purpose, including adsorption of the extract of rice polishings on acid clay, elution with barium hydroxide and successive precipitations with silver nitrate, phosphotungstic acid, platinic chloride and picrolonic acid; and finally decomposition of the picrolonate with hydrochloric acid to yield the crystalline vitamin. Its formula is $C_{12}H_{11}N_4OS$, and its structural formula, as elucidated by Williams, Clarke, and their co-workers is



Thiamine hydrochloride*

Thiamine is soluble in water and in alcohol up to 70 per cent. It is insoluble in fat solvents The pure substance is quite stable in acid solutions and can be sterilized for 30 minutes at 120° C. without appreciable loss of activity; but in alkaline and neutral solutions, the vitamin is rapidly destroyed, because here it is hydrolyzed into its two main components; substances containing the pyrimidine and thiazole rings.

Assay.—The biological test can be based either on curing the polyneuritis (in the pigeon), or causing a resumption of growth (in the rat). When the pigeon is used, it is placed on polished rice until the paralytic symptoms appear; the substance under examination is now given. Where the rat is used, young animals (some thirty days in age) are selected and placed on a synthetic diet devoid of vitamin B_1; for example, purified casein, starch, salt mixture, butter fat, cod liver oil, and autoclaved yeast. The butter fat and the cod liver oil supply vitamin A, the latter also supplies vitamin D, and the autoclaved yeast is a source of the vitamin B complex other than thiamine. The animals stop growing at the end of two weeks. At this point the material under examination is fed.

The biological methods are time-consuming and costly. Several chemical and microbiological methods have been suggested in their place.

One chemical method is based on the measurement of the fluorescence produced by thiochrome, a compound formed when thiamine is oxidized with potassium ferricyanide in alkaline solution.

Several colorimetric methods are based upon the color produced by the coupling of the vitamin with a diazotized aromatic amine (such as sulfanilic acid). A rapid method for the estimation of thiamine is based upon the fact that this vitamin has a powerful accelerating action on the rate of alcoholic fermentation by yeast.

Microbiological methods are having wide applications in the determination of vitamins (and amino acids) and some space should be devoted to them at this stage.

In this instance, the accelerating effect of thiamine on the rate of

* For details of its synthesis, see appendix (p. 568).

alcoholic fermentation is specific. Here baker's yeast is used as the test organism, the medium consists of inorganic salts, glucose and nicotinic acid, the mixture is incubated for three hours at 30° C., and the gas given off is measured.

Another method, of wide applicability, is to select an organism which needs the particular vitamin (or amino acid) for growth, to supply the organism with everything except the substance under examination, and then to add the latter (as a food extract, etc.) and measure the growth response. For example, thiamine may also be assayed by selecting as the test organism, *Saccharomyces cerevisiae*, and using the following medium: casein hydrolysate, sucrose, asparagine, inorganic salts, inositol, biotin, calcium pantothenate, pyridoxine, folic acid and thiamine-free liver and yeast extracts.

TABLE 25.—THIAMINE LOSSES CAUSED BY COOKING (Arnold, Baird and Elvehjem, *Amer. J. Public Health*, **29**, 1344.)

Process.	Meat.	Processing time, min.	Destruction of thiamine, per cent
Frying	Beef round	20	none
	Veal hindquarter	20	45
	Pork ham	15	none
	Smoked ham	15	10
	Pork ham	medium well done	35
		Hours.	
Roasting...	Beef round	2.5	61
	Veal hindquarter	2	58
	Pork loin	1.5	50
Broiling..	Beef round	20 min.	50
Baking	Pork loin	1 hr.	50
Stewing	Beef heart	1 hr.	55
	Beef kidney	45 min.	40

Effect of Heat on Thiamine.—Tables 25 and 26 give some idea of thiamine losses sustained when some common foods are cooked.

Thiamine Requirements.—These are given in Table 13, p. 111.

R. R. Williams and his coworkers state that the average American diet (this is prior to the advent of enriched bread or flour) supplies 0.8 mg. of thiamine per 2500 Calories—a figure below requirements.

An Analog of Thiamine.—Pyrithiamine, a pyridine analog of thiamine (the pyridine displacing the thiazole nucleus), inhibits growth of fungi and has, in general, antithiamine properties. Pyrithiamine administered to mice, not ordinarily susceptible to disease produced by thiamine deficiency, causes them to show marked characteristics of thiamine deficiency; and this state can be cured by the addition of thiamine itself.

The relationship of pyrithiamine to thiamine is analogous to the relationship of sulfanilamide to *p*-aminobenzoic acid (p. 169). In

non-toxic levels, however, pyrithiamine shows little bactericidal power. (For further significance of this experiment, see Chap. 14.)

Biosynthesis of Thiamine.—In the rumen of animals microorganisms synthesize vitamins which may then be utilized—a fact which often makes experimental observations difficult. Najjar and Holt have had some such difficulty in studies on man. They found that on a diet low in thiamine some subjects showed no pronounced deficiency symptoms. It was then discovered that thiamine was being synthesized in the bowel.

Another interesting observation was that when succinylsulfathiazole was administered—a drug which tends to inhibit growth of various microorganisms—synthesis of the vitamin in the bowel was largely curtailed.

TABLE 26.—THIAMINE LOSSES CAUSED BY COOKING. (Aughey and Daniel, *J. Nutrition*, 19, 293)

Food.	Cooking method.	Time of cooking	Destroyed during cooking.
		min.	per cent.
Carrots	Pressure cooker	11–15	0
	Boiled	23 ± 3	0
Potatoes	Baked	63 ± 5	16
	Boiled (pared)	36 ± 5	20
Spinach	Boiled	9	22
Peas, green	Simmered	12	9
	Simmered with soda	12	22
Beans, snap	Boiled	40	18
	Boiled with soda	40	59
Beans, navy	Boiled	83 ± 22	0
	Boiled with soda	53 ± 4	0
Oats, rolled	Double boiler	120	0
Wheat, whole	Double boiler	30	0
	Baked (bread)	45	14
Pork loin (lean portion)	Braised (chop)	13 ± 1	15
	Roasted	43 ± 3 per lb.	43

Enriched Bread (see p. 126).—White bread, made from flour which has been refined and has therefore lost some of its vitamins and minerals, is now made more nutritious by the addition of thiamine, nicotinic acid (p. 159) and iron. Such a bread is known as "enriched bread." Sometimes, but not always, further additions of riboflavin (p. 153), vitamin D (p. 178) and calcium are made.

Government specifications are such that every pound of enriched bread must contain not less than 1 mg. of thiamine, not less than 4 mg. of nicotinic acid, and not less than 4 mg. (nor more than 16 mg.) of iron.

The Council on Pharmacy and Chemistry* of the American Medical Association has this to say of thiamine:

Thiamine is of value in correcting and preventing beriberi.

The consensus of opinion of the students of beriberi is that this disease with its nervous and cardiovascular manifestation is due primarily to an insufficient supply of thiamine. It is probable that in the majority of instances of human beriberi there are also deficiencies of food constituents other than thiamine. There are conditions which probably could be designated as "latent beriberi" . . . .

Thiamine may be cited as of value in correcting and preventing anorexia [loss of appetite] of dietary origin in certain cases.

There are many causes of anorexia, some referable to infections and the reactions thereto, others to organic disorders, and still others related to faulty diet. Where there is no rather obvious cause of anorexia in question, other than a possible dietary one, it is permissible to claim that thiamine may be of therapeutic value when the condition to be treated is due to a deficiency of that vitamin.

The administration of thiamine in excess of that present in the ordinary diet may be advantageous when there are specific conditions indicating interference with proper assimilation of the vitamins.

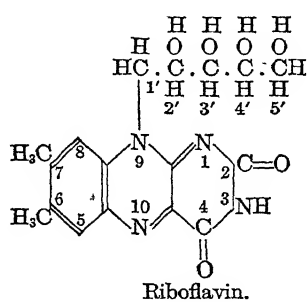
The present status of research on the clinical use of thiamine for specific diseases other than beriberi and for infant feeding, is such that *definite* claims for therapeutic value in relation to such diseases cannot be recognized. Its use may be indicated, however, in such restricted conditions as pernicious vomiting of pregnancy, tube feedings through a jejunal fistula, and the like, because the above permitted statement applies to such conditions and gives an intelligent basis for such therapy.

While it has not been established that thiamine deficiency is the sole cause of conditions described as alcoholic neuritis, the neuritis of pregnancy and the neuritis of pellagra, there is some definite evidence of the value of this vitamin in the treatment of these conditions. Vague representations with respect to the value of thiamine in the treatment of other types of neuritis are not permissible.

Thiamine deficiency in animals is associated with dysfunctions of the heart and of the vascular system. Thiamine is effective in reestablishing the normal function of the cardiovascular system if the dysfunction was caused by thiamine deficiency. Evidence is lacking that thiamine is effective in any other type of heart disease. At times organic heart disease and beriberi heart coexist. Administration of thiamine is justified in these patients.

It appears that there is an increased requirement for thiamine when there is greatly augmented metabolism such as occurs in febrile conditions, hyperthyroidism, or vigorous muscular activity. . . . †

Riboflavin, vitamin B_2, vitamin G, has the formula

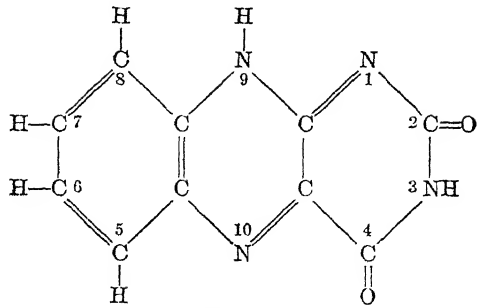


Riboflavin.

Riboflavin, derived from isoalloxazine, is 6,7-dimethyl-9-*d*-1′-ribitylisoalloxazine and

* *New and Nonofficial Remedies,* 1944.

† An enzyme in fresh fish is very destructive of thiamine. Apparently, fresh water fish contain the enzyme, whereas salt water fish do not. The Chastek paralysis, occasionally seen in the fox, is due to the inclusion in the diet of fresh or frozen fish of certain species.



Isoalloxazine.

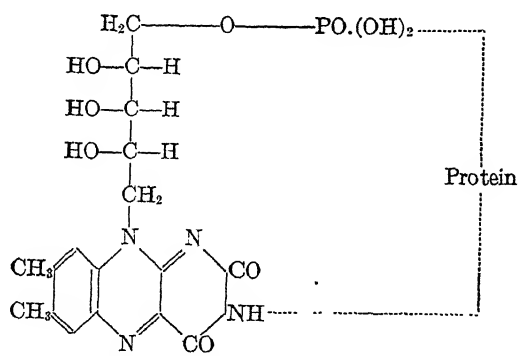
has a structure which exhibits several groups: a pyrimidine (1,2,3,4); azine (9,10); benzene (5,6,7,8); and the sugar ribose attached at position 9.

The vitamin properties of the compound are apparently dependent upon these structural components. For instance, by substituting arabinose for ribose, the resulting compound is much less active.

The crystalline, orange-yellow, water-soluble material is fairly heat-stable, and stable to air (and oxygen), but very photolabile, so that solutions are rapidly affected when exposed to light. The solutions are much more stable in acid than in alkaline media.

The solution, greenish-yellow in color, has a greenish-yellow fluorescence. Under ultraviolet rays the fluorescence becomes much more intense, and this is made the basis for an estimation of riboflavin.

Warburg's respiratory yellow enzyme (p. 392) is riboflavin connected to a protein by means of phosphoric acid.



Yellow enzyme (Warburg).

Even more important than the yellow enzyme are flavin compounds which make up the amino acid oxidase (p. 393) and xanthine oxidase (p. 393) because these have more obvious functions and are more widely distributed.

As showing how closely related the vitamin is to these enzymes, Elvehjem has shown that the enzymes are decreased in activity on a

* This compound, dissociated from the protein, may be regarded as a nucleotide (p. 86), in which the purine group is replaced by isoalloxazine.

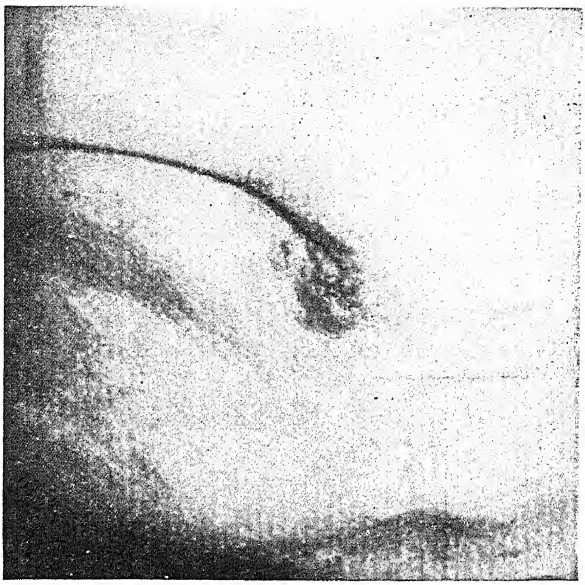


Fig. 41.—Cheilosis. The illustration portrays one of the lesions seen in patients whose daily dietary intake of riboflavin is insufficient to maintain normal nutrition. Typical oral lesions of riboflavin deficiency consist of fissures at the angles of the mouth, a reddened, denuded appearance of the lips, and flattening of the papillae of the tongue which has a characteristic magenta-red hue. These lesions respond to specific therapy with riboflavin. *This photo is reproduced by courtesy of Drs. G. B. Myers and I. M. Clapper, Detroit, Michigan.* [Therapeutic Notes, March, 1943.]

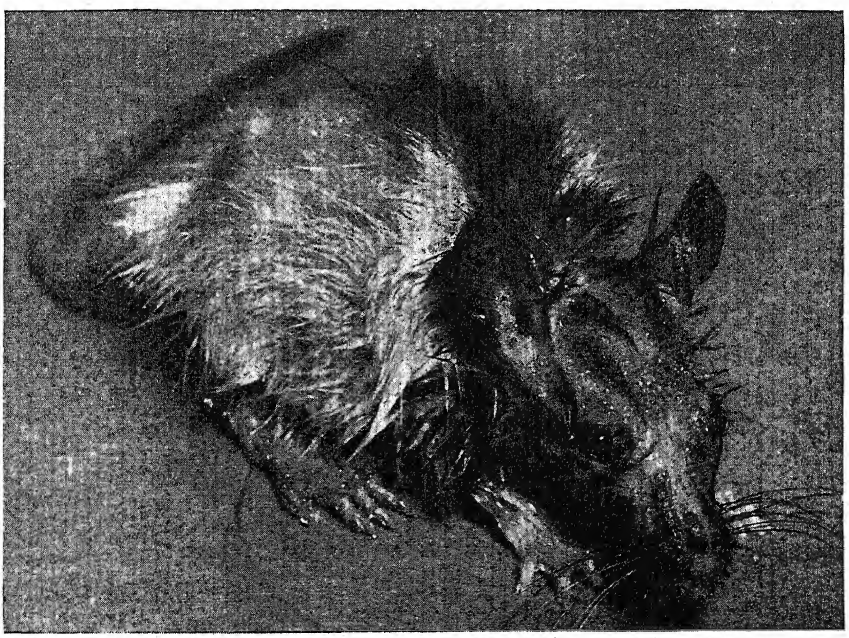


Fig. 42.—Riboflavin deficiency. (Research Laboratories, S. M. A. Corporation.)

diet deficient in riboflavin. The conclusion is obvious: one important function of the vitamin is to supply the "building stones" for the formation of such enzymes.

In riboflavin deficiency growth is arrested; but this is not at all characteristic.

The disease due to lack of riboflavin—ariboflavinosis—is characterized by cheilosis (Fig. 41), a reddening of the lips, with lesions in the angles of the mouth; glossitis, inflammation of the tongue; seborrheic dermatitis, waxy accumulations in the skin; keratitis, ocular lesions, a burning and roughness of the eyes; and "sharkskin," a roughening of the skin at the mouth and nose. (Also Fig. 42.)

Fig. 43.

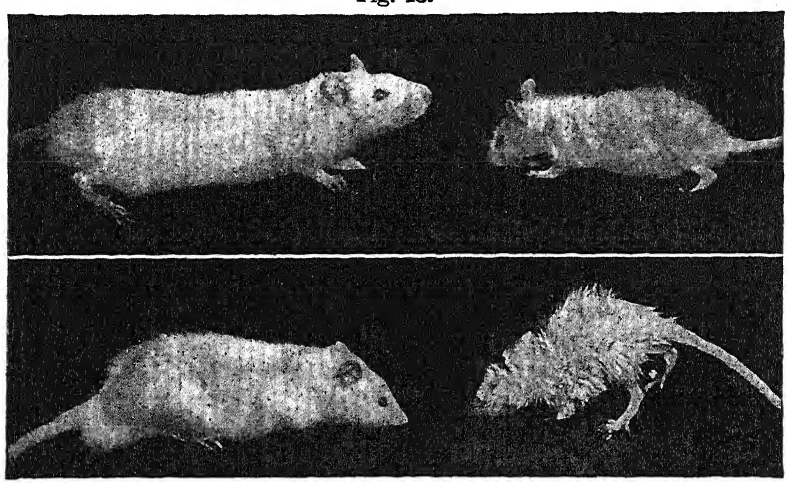


Fig. 44.

Fig. 43.—The rat on the right was fed the low riboflavin carbohydrate ration for twelve weeks. The rat on the left received the same ration with intake restricted to that of the former plus 100 micrograms riboflavin per day. (Shaw and Phillips, *J. Nutrition*, **22**, 345.)

Fig. 44.—The rat on the right was fed on the high fat ration for eight weeks. The rat on the left received the same ration with intake restricted to that of the former plus 100 micrograms riboflavin per day. (Shaw and Phillips, *J. Nutrition*, **22**, 345.)

While it seems definite that riboflavin is a vitamin needed by man, the symptoms of deficiency are usually complicated by other factors.

The effect on rats of riboflavin-deficient diets is shown in Figs. 43 and 44.

The two rations used were deficient in riboflavin. The first consisted of the following:

	Per cent.
Dextrin	72
Alcohol extracted casein	18
Lard	3
Corn oil	3
Salts	4
	100

	Per 100 gm. ration.
Thiamin	300 μg.
Pyridoxine	300 μg.
Nicotinic acid	5 mg.
Pantothenic acid	2 mg.
Choline	200 mg.

The second ration was identical with the first with the exception that part of the dextrin was replaced by lard. The fat content of this ration was 39 per cent. Twice weekly, these rations were further

TABLE 27.—FOOD SOURCES OF RIBOFLAVIN. (Daniel, *Food and Life.*)

Type of food.	Excellent sources.	Good sources.	Fair sources.
Animal products	Liver, kidney, heart, lean muscle meats, eggs, cheese, dried (whole or skim), condensed, and evaporated milk.	Fresh (whole or skim) milk, buttermilk, whey.	
Vegetables	Turnip tops, beet tops, kale, mustard greens.	Peas, lima beans, spinach, water cress, collards, endive, broccoli, green lettuce, cabbage, cauliflower, carrots, beets.	
Fruits		Avocados, prunes, mangoes, peaches.	Bananas, cured figs, grapefruit, oranges, apricots, guavas, papayas, muskmelons, apples.
Seeds	Germ portion of wheat, rice polishings, peanuts, soybeans.	Whole-grain wheat, dried legumes.	

supplemented by feeding 3 drops of haliver oil which contained 1 mg. of added synthetic α-tocopherol per 3 drop dose. Small quantities of ration were mixed as needed. The rations were stored in the refrigerator and fed daily to prevent rancidity.

Distribution.—Riboflavin is widely distributed, as can be seen from Table 27.

Assay.—The biological method, using the rat, can be employed. One such method uses the following diet (Table 28):

TABLE 28.—RIBOFLAVIN-FREE BASAL RATION K_{21}. (Wagner, Axelrod, Lipton and Elvehjem, *J. Biol. Chem.*, **136**, 367.)

Dextrin	65 parts
Casein (Labco)	18 "
Salts I	4 "
White corn	6 "
Butter fat	3 "
Cod liver oil	2 "
Corn oil	2 "
Thiamin	200 γ per 100 gm.
Pyrixodine	200 " " 100 "
Nicotinic acid	2500 " " 100 "
Riboflavin-free liver extract	≈4% of starting material

Rats placed on this diet decline in weight. The food to be tested for riboflavin can now be incorporated.

The riboflavin-free liver extract requires special preparation. While it is practically devoid of riboflavin, it still contains many of the needed constituents of the vitamin B complex.

Another method of estimating riboflavin is to measure its fluorescence under ultraviolet rays.

Still another method is a microbiological one. The lactic acid group of bacteria are unable to synthesize riboflavin and need it for development. This, therefore, becomes one method of assaying the vitamin. This microbiological method uses *Lactobacillus casei* ϵ as the test organism. The amount of flavin added to the nutrient solution is proportional to the growth of the organism.

Because of rapidity of execution, and also because of comparative accuracy, the microbiological and the fluorometric methods are widely used.

Human Requirements.—Human requirements for riboflavin are stated to be between 2–3 mg. per day (see Table 13, p. 111).

Various surveys have shown that many American families of the poorer group do not get this daily amount. And yet here too, as in the case of thiamine (p. 152), it is possible that some riboflavin may be synthesized by microorganisms in the large intestine.

In ruminants this type of bacterial synthesis (of some vitamins, at least) is so strong, that neither a riboflavin nor a thiamine deficiency can be produced experimentally.

Actual experiments on normal adult males show the average excretion of riboflavin to be from 800–1200 micrograms* per day.

The question of whether any extra energy may be made available by an extra supply of riboflavin—and several other vitamins such as thiamine, nicotinic acid, pyridoxine and pantothenic acid—has been answered in the negative by Keys as a result of elaborate experiments with United States Army men.

Keys has also studied the effect of restricted intake of some of the vitamins. He finds, for example, that "normal young men suffer no physiological handicap from subsistence for at least 5 months on a diet providing 0.31 mg. of riboflavin per 1000 Cal (0.99 mg. per day for 3150 Cal.)." This is considerably less than the amount recommended by the National Research Council (2.7 to 3.3 mg.).

However, five months is far from a lifetime; and much can happen of a *gradual* kind over the course of years. (Recall Chittenden's experiments, for example, p. 110.)

Enriched Bread (see p. 126).—In addition to thiamine, nicotinic acid and iron, it has been proposed that 1.2 mg. of riboflavin per pound of flour be incorporated.

The Council on Pharmacy and Chemistry† of the American Medical Association has this to say of riboflavin:

> Riboflavin is recognized as a specific in the treatment of certain characteristic lesions of the tongue, the lips, and the face. The symptoms may be described

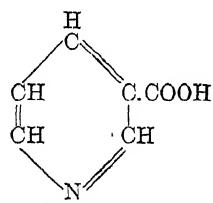
* The microgram is one-thousandth of a milligram.

† *New and Nonofficial Remedies*, 1944.

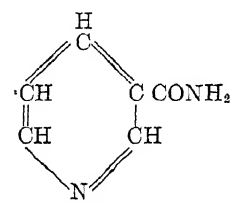
briefly as follows: A typical glossitis may often be observed before other signs of riboflavin deficiency are present. In contrast to the glossitis of pellagra, the tongue is clean, the papillae are flattened or mushroom-shaped rather than atrophic, and the color is definitely purplish-red or magenta instead of being scarlet as in nicotinic acid deficiency As the disease progresses, the lips become reddened, then shiny and denuded, with maceration and fissuring at the angles of the mouth (cheilosis). Frequently, seborrheic follicular keratoses occur at the nasolabial folds and even over the nose and forehead The above symptoms are promptly alleviated by the administration of adequate amounts of riboflavin.

Riboflavin deficiency is responsible for certain ocular manifestations characterized by itching, burning and a sensation of roughness of the eyes (keratitis), accompanied by mild photophobia. A deficiency of riboflavin results in a retardation of growth, but it must be borne in mind that riboflavin is no more important in contributing to normal growth than any one of the other vitamins, the essential mineral elements, or amino acids

Niacin,* nicotinic acid, nicotinic acid amide, P-P factor, is the curative factor in canine blacktongue (the analogue of pellagra in man) and in human pellagra.



Nicotinic acid.

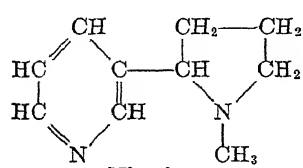


Nicotinic acid amide or nicotinamide.

This discovery we owe very largely to Elvehjem and his associates.

Pellagra is a disease which shows itself by "three d's," as Harris puts it—by a dermatitis, diarrhea and dementia (Fig. 45). In the southern states, where the disease is still too prevalent, it is brought about by a diet largely of "three m's"—maize meal (corn meal), molasses and meat (fat pork). Working primarily as a clinician, Goldberger had proved conclusively that pellagrins could be cured by feeding them more fresh meat, eggs and milk—substances now known to contain nicotinic acid or its derivative.

Nicotinic acid, though comparatively new in the vitamin field, has been a well-known substance to chemists since 1867. One method of obtaining it is by the oxidation of nicotine with nitric acid or potassium permanganate.†



Nicotine.

In 1911, long before nicotinic acid was shown to be a cure for pellagra, Funk had actually isolated the substance from rice bran con-

* This name has been employed in the place of nicotinic acid to prevent confusion with nicotine.

† Unfortunately, smokers cannot convert the nicotine which they inhale into nicotinic acid!

centrates and had shown that it had no effect in curing polyneuritis in pigeons.

The acid as well as the amide is soluble in water and alcohol.

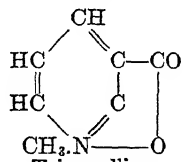
Function.—Coenzyme I (p. 87) and coenzyme II (p. 87), both of which are involved in carbohydrate metabolism (p. 396), are derivatives of nicotinic acid amide. The need for the substance now becomes apparent. Elvehjem has indeed shown that in dogs suffering from a deficiency of nicotinic acid there is an appreciable decrease in the amount of coenzyme I in liver and muscle (coenzyme II was not estimated). The change in blood is slight, if any.

Fig. 45.—A negress from South Carolina with the typical "pellagra glove" symptom. (From Lavinder and Babcock.)

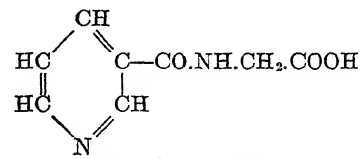
Structure and Physiological Activity.—The relationship of structure to physiological activity may be illustrated with nicotinic acid. The alpha and gamma isomers of the acid (picolinic acid and isonicotinic acid) are inactive. Inactivity also results from the substitution of a methyl or a carboxyl group for one of the ring hydrogens, or by the addition of a methyl group to the ring nitrogen. The replacement of the carboxyl group of nicotinic acid by a sulfonic or a cyano group, or the removal of the carboxyl group—giving rise to pyridine—also destroyed all activity.

Compounds other than nicotinic acid or its amide which are physiologically active (in varying degrees) are so because they are apparently converted to the acid or amide in the body. Several such examples are ethyl nicotinate, β-picoline (a methylpyridine) and nicotinuric acid.

Metabolism of Nicotinic Acid.—When fed nicotinic acid, man, the dog and the rat excrete in the urine trigonelline (the methyl betaine of nicotinic acid) and possibly some nicotinuric acid (the combination of the acid with glycine).



Trigonelline.



Nicotinuric acid.

Dogs maintained on a diet low in nicotinic acid retain much of it when the acid is subsequently offered them. This also applies to humans.

In one biological method, based on curing blacktongue in dogs,* the animal is given a basal ration of yellow corn, purified casein, cottonseed oil, codliver oil, calcium phosphate, calcium carbonate, sodium chloride, thiamine and riboflavin. On this diet blacktongue is regularly produced. The food (or other test object) is then added to test its curative properties.

One satisfactory colorimetric method is based upon the action of cyanogen bromide on the acid—which causes a breakdown of the pyridine nucleus—and coupling the product with aniline, yielding a yellow compound which can be measured colorimetrically.

Several microbiological methods have been suggested. One such method makes use of *Lactobacillus arabinosus* as the test organism. The medium consists of a casein hydrolysate, together with glucose, tryptophan, cystine, sodium acetate, inorganic salts, purine and pyrimidine bases, thiamine, calcium pantothenate, pyridoxine, riboflavin and biotin. The mixture is incubated for 72 hours at 30° C. The measurement of growth response—after the test object is added—is determined by titrating the acid produced.

Food Sources of Nicotinic Acid.—See Table 29.

An excellent source of nicotinic acid is yeast. Good sources are liver and lean meats. Other sources of a practical kind are leafy green vegetables, tomatoes and milk.

For recommended daily allowances, Table 13, p. 111.

Exact requirements for human beings are not easy to determine. When an excess of nicotinic acid is offered, some is excreted not only as the acid but also as trigonelline and nicotinuric acid.

That pellagra may develop on a diet largely of cornmeal and patent flour becomes probable when we consider that 100 gm. of these foods contains not more than 1 to 1.5 mg. of nicotinic acid, whereas the daily requirements vary from 15–23 mg.

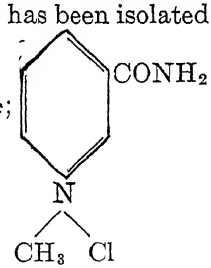
Claims that urinary pigments—porphyrins, etc.—are characteristic excretion products of pellagrins, and that these pigments disappear with treatment (by niacin) have not been confirmed.

* The rat apparently is not susceptible to deficiencies due to nicotinic acid. The evidence points to the fact that the rat can synthesize the acid if, and when, the animal is in need of it.

TABLE 29.—FOOD SOURCES OF NICOTINIC ACID. (*Bull. Lederle Lab*, 10, 31)

	Milligrams per 100 grams of fresh tissue
Pork liver	19–30
Beef liver	16–23
Veal liver	13–20
Beef kidney	6– 8
Beef heart	7– 8
Beef round	7– 9
Fresh ham	7– 8
Smoked ham (tenderized)	4– 8
Pork loin	6– 7
Chicken—white meat	7
Chicken—dark	6
Salmon	7
Rye—whole	13
Rye flour—dark	12
Rye middlings	17
Whole wheat flour	5.2
Cornmeal	0 6
Milk	0 08
Rolled oats	1 40
Barley flour	5 7

A fluorescent material, known as F_2, which is found in the urine after the administration of nicotinic acid or its amide has been isolated and shown to be Chloride of N′-methylnicotinamide;



Using liver slices, the amide, but not the acid, was converted to this methylated derivative. Substituting kidney and muscle slices in the place of those of liver gave negative results.

Antagonistic Action of an Analogous Compound.—Largely as the result of the work of Woolley, it is becoming apparent that vitamin deficiency diseases may be developed by certain compounds analogous in structure to the curative agents (for further discussion, see Chap. 14). As an example, Woolley has shown that 3-acetylpyridine—where the —COOH in nicotinic acid has been exchanged by —$COCH_3$—gave rise to typical nicotinic acid deficiency in mice (a species not usually susceptible to nicotinic acid deficiency when produced by the usual nutritional means). The disease could be cured by the addition of the vitamin (or its amide) to the diet. However, the 3-acetylpyridine had no such inhibitory effect in the growth of certain microorganisms.*

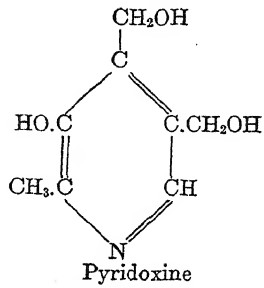
The Council on Pharmacy and Chemistry† of the American Medical Association has published the following in reference to nicotinic acid and nicotinic acid amide:

* Bovarnick has obtained nicotinamide by heating asparagine (p. 374) and glutamic acid (p. 52). Such a heated mixture, she has shown, can be substituted for the vitamin as a growth factor for *Bacterium dysenteriae* and other growth organisms.

† *New and Nonofficial Remedies*, 1944.

Nicotinic acid and nicotinamide are recognized as specific only in the treatment of pellagra. Their administration in appropriate doses lead to the disappearance of all alimentary, dermal, and other lesions, characteristic of the disease, . . . and to a profound improvement in the mental symptoms when the latter are the result of an inadequate intake of nicotinic acid and nicotinamide. These compounds are without influence upon the polyneuritis or cheilosis so frequently observed in pellagrous patients. In such cases it may be necessary to insure the presence in the diet of foods rich in thiamine or riboflavin, or to administer these vitamins.

Pyridoxine, vitamin B_6.—A deficiency of this vitamin in young rats results in the development of a dermatitis, with swelling and



Pyridoxine

[2-methyl-3-hydroxy-4,5 (hydroxymethyl) pyridine]

edema [especially located in the ears, nose, and digits of the paws (Figs. 46, 47)]. This skin disturbance, at first thought to resemble pellagra, cannot be cured with nicotinic acid.

Fig. 46.—Vitamin B_6 deficient rat. (*Merck Report*, July, 1939.)

Pyridoxine has been isolated (from rice bran, for example) and synthesized. The hydrochloride, a white, crystalline substance, is soluble in water, less so in alcohol and acetone and insoluble in ether. The aqueous solutions (*p*H about 3) are comparatively stable, and sterilization (120° C. for 20 minutes) does not destroy the vitamin.

Some workers in the field have associated the dermatitis in pyridoxine-deficient animals with a type of dermatitis known as "acrodynia."* It is believed by many that this "acrodynia" as seen in rats is not, in reality, related to human acrodynia.

The claim has further been made that the type of dermatitis which develops in the absence of pyridoxine can be cured by the "essential fatty acids" of Burr (p. 118) or by a rice bran concentrate containing

* Acrodynia is defined as an eruptive disease, marked by increased sensibilities of the soles and the palms, with pricking sensations in them and rheumatoid pains in the hands and the feet. There is an erythematous eruption followed by a brown pigmentation.

pyridoxine and a second "accessory factor." Pyridoxine itself, according to these experiments had only a temporary effect on "rat acrodynia" in the absence of the "accessory factor."

In contrast to the dermatitis observed in rats, chicks fed a diet deficient in pyridoxine show no such symptom. On the other hand, they

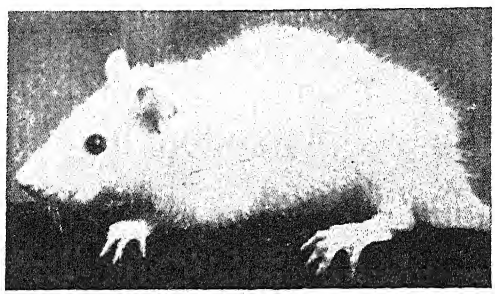


Fig. 47.—Rat cured by vitamin B_6. (*Merck Report*, July, 1939.)

grow very slowly and develop convulsions and other nervous symptoms (Fig. 48).

Assay.—The tentative unit for pyridoxine represents the minimum amount of this vitamin which will cure the type of skin disease known as acrodynia* in standardized rats in twenty-one days when on a

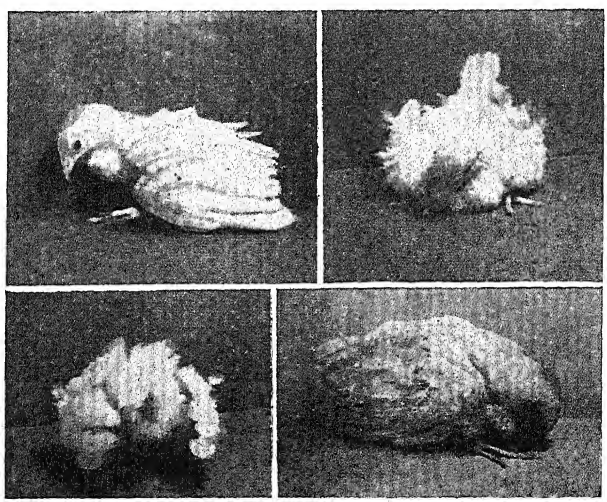


Fig. 48.—Positions assumed by pyridoxine-deficient chicks. (Lepkovsky and Kratzer, *J. Nutrition*, **24**, 515.)

pyridoxine-deficient diet. The vitamin is determined by the use of this biological method.

A microbiological method which has been suggested depends upon the influence of pyridoxine on the growth of *Streptobact. plantarum.*

* One such pyridoxine-deficient diet consists of sucrose, purified casein, salt mixture, crisco, liver filtrate free from pyridoxine, cod liver oil, agar, thiamine and riboflavin.

One of several color reactions which have been proposed depends upon the formation of a blue indophenol compound when pyridoxine and a quinone derivative are brought together.

Requirements.—Claims have been made that the vitamin is essential not only for the rat, but for the chick, pigeon, dog and pig.

As to human requirements, little can be said at this stage. Whether pyridoxine is required by man is debatable, for no definite pyridoxine deficiency has so far been observed. But experiments with animals suggest that if the vitamin is essential to humans, some 2 mg. per day, comparable in amount to the needs for thiamine, would meet requirements.

Pyridoxine is essential for the growth of excised plant roots, for certain strains of yeast and for certain fungi (for example, *Graphium ulmi*, the organism which causes Dutch elm disease).

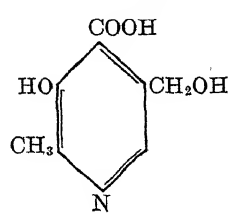
Occurrence.—Good sources are rice bran, liver, yeast, cereals, legumes and milk.

Source.	*Pyridoxine: mg. per 100 g. of food.*
Whole wheat	0.46
Many meats (fresh basis)	0 4–0.7
Fresh vegetables	0 1
Milk	2 mg. (per quart)

Function.—Working with rats, Lepkovsky and others have succeeded in showing that pyridoxine plays a rôle in the metabolism of tryptophan. From the urine of rats on a pyridoxine-deficient diet, xanthurenic acid (4,8-dihydroxyquinoline-2-carboxylic acid) was isolated. This acid has already been recognized as a product of tryptophan metabolism. Furthermore, in experiments with swine, it was shown that the amount of kinurenine, another metabolic product of tryptophan (p. 370), excreted diminished with the addition to the diet of pyridoxine.

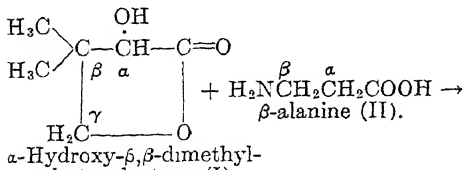
Another interesting development dealing with the function of this vitamin was to show that pyridoxine is connected with the coenzyme in bacteria which facilitates the conversion of tyrosine to tyramine (p. 232)—a decarboxylation reaction.

Huff and Perlzweig have isolated from the urine a fluorescent substance obtained after the ingestion of pyridoxine. It is 2-methyl-3-hydroxy-4 carboxy-5-hydroxymethylpyridine



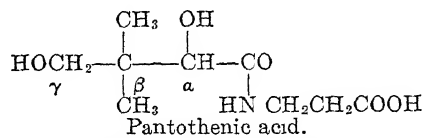
Possible Relation to Alanine.—Snell and Girard have shown that alanine can replace pyridoxine as a growth factor for *Streptococcus lactis* R. Is alanine, then, a precursor of pyridoxine?

Pantothenic acid, filtrate factor, chick antidermatitis factor, is represented by formula III. The isolation of pantothenic acid from liver extracts led to a chemical study of the substance. A butyrolactone (I) and β-alanine (II) were obtained from it. The synthesis was accomplished by condensing I and II.



α-Hydroxy-β,β-dimethyl-γ-butyrolactone (I)

β-alanine (II).



Pantothenic acid.
[N-(α,γ-dihydroxy-β,β-dimethylbutyryl)-β-aminopropionic acid]
(III).

Working with many types of tissues, R. J. Williams and his coworkers showed that they all contained a "something" which had a stimulating effect on the growth of certain strains of yeast. This "something" was finally isolated and given the name "pantothenic acid" (meaning "from everywhere").

On a diet deficient in pantothenic acid the rat will exhibit poor growth, dermatitis and a graying of hair.

The acid is most easily isolated in the form of its calcium salt. The dextrorotatory form of the salt is used, for the levo modification shows little biological activity. This calcium salt is a white, crystalline powder, soluble in water, the aqueous solution being alkaline (*p*H 8.5). Autoclaving causes decomposition, and sterile solutions may be obtained by Berkefeld filtration.

On a vitamin B complex-free diet, but with daily supplements of thiamine, riboflavin and pyridoxine and a daily addition of 10 micrograms of pantothenic acid to rat A (Fig. 49), rat B showed signs of graying at the end of three weeks.*

This biological assay is not only time-consuming but not sufficiently quantitative. A more satisfactory procedure is the microbiological method, using *Lactobacillus casei* as the test organism and measuring the increase in organic acid production when panthothenic acid (or substances containing it) are added to the medium.

Requirements.—Little can be said at present as to human requirements of panthothenic acid, assuming that the acid is needed by humans. Excretion studies on man (using *Lactobacillus casei* as the test organism) point to an average daily elimination of 3–4 mg.,

* There are differences of opinion as to whether pantothenic acid is the sole anti-graying factor. Some claim that the anti-graying potency of natural products—rice bran, liver, yeast—is more powerful than the acid. Biotin (p. 167) and para-aminobenzoic acid (p. 169) have been suggested as additional factors.

which would indicate that this amount, at least, would have to be replaced in the diet.

Sources.—Yeast and liver are two of the richest sources.* Other good sources (in decreasing order of value) are eggs, sweet potatoes, lean beef, whole milk and tomatoes.

Biotin, anti-egg white injury factor, vitamin H, coenzyme R. The word "biotin" is an offshoot of the older word "bios," which was coined by Wildiers more than forty years ago. Wildiers grew yeast on synthetic media and discovered that growth was retarded unless some "x substance" found in beer wort and in *growing* cultures of yeast was present.

One function of biotin became clear after certain studies involving egg white as a source of protein in rat diets. Such a diet gives rise to a severe dermatitis. This was traced to the presence in the egg white

A B

Fig. 49.—Effect of pantothenic acid deficiency. (Research Laboratories, S. M. A. Corporation.)

of a protein known as *avidin.*† It is now believed that in an otherwise wholesome and complete diet, avidin, if present, combines with biotin in the intestinal tract and thereby neutralizes one function of biotin—which is to prevent the dermatitis. Of course, if the amount of avidin is limited and there is an abundant supply of biotin, the dermatitis is avoided.‡

Metabolism of Biotin.—Normal human beings eliminate from 20 to 50 micrograms of biotin per day. The biotin seems to be in a free, rather than in a combined, state.

* An intriguing possibility has been raised by workers on royal jelly—a secretion of the pharyngeal glands of the honey-bee—which is the richest known source of pantothenic acid. For the first two days after hatching all female larvae receive a diet of royal jelly. During the third day the diet of the larvae that are to become workers is changed, while the queen caste continues to receive royal jelly. Does pantothenic acid contribute to the formation of a queen?

† So far the only source of avidin is egg white.

Besides avidin, egg white also contains a protein, lysozyme, which lyses or dissolves such microorganisms as *Micrococcus lysodeiktieus* by hydrolyzing a mucoid contained in the bacterial membrane.

‡ There is evidence for the belief that avidin formation is in some way connected with the reproductive function of the ovary.

Intestinal bacteria synthesize biotin quite readily, just as they do vitamin K (p. 191). In fact, because of this, a biotin deficiency is difficult to produce. One way is to feed sufficient egg white (or avidin) to neutralize the growth effects of the biotin.

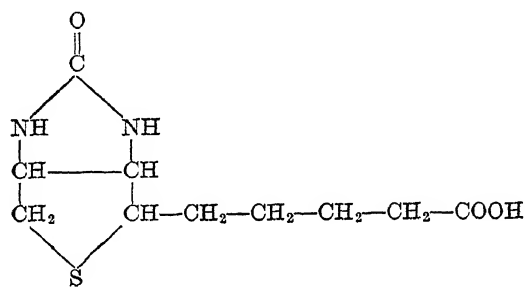
The amount of biotin synthesized by intestinal bacteria may be sufficient so that a source of the vitamin in foods becomes unnecessary.

Assay.—One method of assay is to test the effect of biotin (or its equivalent in foods) on rats suffering from egg white injury.

More rapid methods are microbiological in character, and depend upon the growth requirements of certain strains of yeast and bacteria.

Chemistry.—Kogl isolated the methyl ester of biotin from egg yolk, and du Vigneaud obtained the same substance from a liver concentrate. The hydrolysis of the ester gives the free biotin, with a formula $C_{10}H_{16}O_3N_2S$.

Biotin is a monocarboxylic acid with two rings. The acid is valeric acid, $CH_3CH_2CH_2CH_2COOH$. One ring is of the imidazole variety (p. 53) and the other is a thiophene derivative. Its complete structure is



and the vitamin has been synthesized.

Antibiotins.—Avidin may be regarded as an "anti-vitamin," opposed specifically to biotin. This "anti-vitamin" is the first of a group of such substances to be observed, and it suggests that "anti-vitamins" play an important physiological rôle in regulating the action of the vitamins themselves.

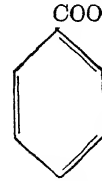
Antibiotin activity is shown by several analogs of biotin. For example, du Vigneaud has shown that desthiobiotin (biotin in which the S atom is replaced by 2H atoms) has antibiotin activity. This also applies to biotin sulfone (biotin in which the S has been oxidized). 0.03 micrograms of the sulfone will reduce the growth of a culture of *L. casei* containing 0.0002 microgram of biotin to that of a culture containing 0.0001 microgram.

Just as *p*-aminobenzoic acid, if added in sufficient amount, overcomes the inhibitory effect of sulfanilamide (p. 285), so the inhibitory effect of these antibiotins may be overcome by the addition of more biotin.

Source.—The liver and the kidney are very rich sources of biotin.* Smaller quantities are found in egg yolk, tomatoes and carrots.

* It has been known that hepatic tumors may be developed by the administration of the dyestuff, butter yellow, to rats. The addition of riboflavin served, to a

Para-aminobenzoic Acid.—Ansbacher noticed that a mouse achromotrichia (lack of pigment in hair) could be cured by feeding rice polishings or by the addition to the diet of para-aminobenzoic acid.



Para-aminobenzoic acid.

Ansbacher further claimed that the acid was an essential constituent for the growth of chicks and was needed by rats for the maintenance of a normal fur coat.

Relation to Sulfanilamide.—Another interesting property of *p*-aminobenzoic acid is its inhibitory effect on the action of sulfanilamide on streptococci. The bacteriostatic properties of the sulfanilamide group towards bacteria (p. 283) are inhibited.

Woods, the discoverer of this antisulfanilamide property of the acid, developed the theory that sulfanilamide competes with *p*-aminobenzoic acid "at the surface of an enzyme which is essential for growth"; and suggested that "normally *p*-aminobenzoic acid is a constituent of this hypothetical enzyme but can be displaced by sulfanilamide," a process which, if it occurs, prevents the enzyme from functioning, and so prevents growth.

Fildes suggested that *p*-aminobenzoic acid is a necessary food for all organisms which are inhibited by sulfanilamide.

These views, taken in conjunction with the similarity in structure between *p*-aminobenzoic acid and sulfanilamide, have been a great incentive in furthering the work relating chemical structure to physiological action. (For further details see Chap. 14.)

Extracts obtained from yeast have the power of preventing achromotrichichia in rats and of inhibiting the action of sulfanilamide on hemolytic streptococci. The conclusion has been drawn that the substance responsible for these effects—present in yeast and part of the vitamin B complex—is para-aminobenzoic acid, a compound which, incidentally has been familiar to the organic chemist for many years.

p-Aminobenzoic acid has been isolated from yeast. It probably occurs there in the form of a peptide with glutamic acid.

Anti-gray Hair.—Ansbacher has been the chief exponent in claiming the anti-gray hair properties of the acid. He has stated, for example, that the acid would cure the graying of rats maintained on a vitamin B-deficient diet and supplemented with thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid, inositol and choline chloride. These findings were not confirmed by Emerson.

large extent, as a protective influence against this tumor development. When, however, biotin was added, the protective influence largely disappeared. The biotin had a "procarcinogenetic effect."

Pigmentation.—Para-aminobenzoic acid is one of a number of the more recently discovered vitamins which are alleged to function in the process dealing with the pigmentation of the skin. Two other vitamins which have found themselves in this category are pantothenic acid (p. 166) and biotin (p. 167). It is possible that not merely one of these factors, but all three of them play some part in what appears to be a very complicated problem.

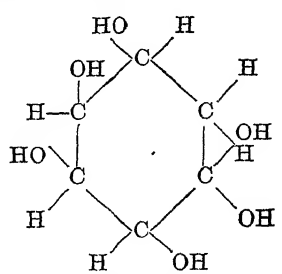
There is, at present at least, no "anti-gray hair vitamin" in the sense that the layman might interpret the phrase: a vitamin which converts gray hair into black hair. In the light of present knowledge, it is possible that diet and gray hair have some causal relationship, but it is highly improbable that diet alone is the sole factor.

It is true that a gray-haired condition has been produced in rats, dogs, guinea-pigs and silver foxes on certain types of deficient diets; but it seems a far cry from this experimental procedure to the process of aging as it occurs in human beings.

Assay.—Several microbiological methods have been suggested. One such method makes use of the growth effects on *Neurospora crassa.* Several colorimetric methods have also been suggested. In one of them, diazotized thiamine is coupled with *p*-aminobenzoic acid to yield a colored product.

Inositol, mouse anti-alopecia* factor. We owe to Woolley the addition of inositol to the group of vitamins known as the "vitamin B complex." Working with young mice, and using a synthetic diet containing all the known vitamins, Woolley discovered that his mice failed to grow and that their hair was affected. The addition of pantothenic acid—the absence of which may also give rise to hair changes—proved useless. Neither was the addition of biotin or para-aminobenzoic acid any better. Cures were observed by the addition of phytin, obtained from cereal grain, or inositol,† isolated from liver.

Inositol is required by certain yeasts for normal growth. The mouse requires it, as Woolley has shown. It has also been claimed that a



Inositol
(Hexahydroxycyclohexane).

"spectacled eye" condition in rats can be cured by inositol; and it seems fairly clear that the substance has a definite growth-promoting action in chicks.

* alopecia = baldness.

† In plants inositol is found in combination with phosphoric acid. Phytin is the calcium-magnesium salt of such a combination.

The contradictory results occasionally obtained—with mice, for example—have been shown, here again, to be due to the ability of the microorganisms in the intestines to synthesize inositol, sometimes in amounts sufficient to prevent the development of alopecia.

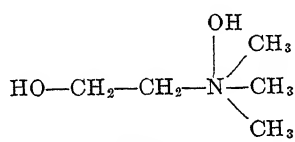
Unlike the ordinary rat, the cotton rat needs inositol for normal growth. This is also true, to some extent at least, for guinea pigs and hamsters.

Occurrence.—Inositol is found in muscles, liver, kidneys, brain and other animal tissues, and also in various fruits and vegetables.

Inositol has been isolated from the phosphatides of the tubercle bacillus and has been found in the cephalin fraction of the brain and spinal chord. It has also been found in the phosphatide of the soybean; Woolley has given it the name *lipsitol*, a compound containing 16 per cent inositol, besides galactose, fatty acids, phosphoric acid and ethanolamine.

Assay.—Inositol stimulates the growth of yeast cells (*Saccharomyces cerevisiae*) in a special medium.

Choline as a constituent of lecithin has already been discussed



Choline.

under the phosphatides (p. 35). The part it plays in fat metabolism and its importance as a methylating agent will be referred to presently (p. 337). Here we are concerned with choline as a member of the vitamin B complex.

It has been shown that in addition to manganese, an organic factor was needed to prevent perosis* in chicks. This factor, present in 95 per cent alcoholic extract of dried liver, was identified as choline by Jukes.

Choline also stimulates growth in turkeys, chicks and dogs; and it has been suggested that the substance is needed for lactation and growth of rats.

Occurrence.—The richest source is egg yolk. Liver and kidney are other good sources. In cereal grains the choline is found largely in the germ.

Folic Acid.—Some lactic acid bacteria require an essential factor which is found in liver extracts. Using *Streptococcus lactis* R. as a test organism, it has been shown that in addition to liver, kidney, mushroom, yeast, and particularly green leaves and grass contain the factor. The name "folic acid" (folium = leaf) has been given to the substance.

Little is known at present about the folic acid requirements of higher animals.

* Perosis is a shortening and thickening of the bones and is often accompanied by a "slipped tendon."

Folic acid has not been isolated as yet,* but concentrated forms, isolated from spinach, show an absorption spectrum resembling that of xanthopterin.

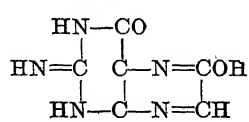
In this connection it is interesting to note that this same xanthopterin, known among other things as an antianemia factor for fish and probably other animals—possibly even related to an antianemia factor vitamin B_c—increased the formation of folic acid when incubated with rat liver.

TABLE 30.—VITAMIN B CONTENT OF A FEW TYPICAL FOODS. [(Elvehjem, *Handbook of Nutrition* (1943), p. 216).]

Milligrams per hundred grams (edible portion).

Food.	Thiamine.	Riboflavin.	Nicotinic acid	Pantothenic acid.	Pyridoxine.
Apples	0.025	0 050	0.500	0.050	
Bananas	0.040	0.080	0 600	0 070	
Bread:					
White (unfortified)	0.070	0.100	0 800	0 400	0 300
White (fortified)	0 280	0 14	1 500	0.400	0 300
Cabbage	0 060	0 050	0 290	0.225	0 290
Carrots	0 050	0 100	1 500	0 210	0 190
Cheese	0 030	0.500	...	0 350	
Cornmeal	0 200	0 150	1.500	0.800	
Eggs	0.250	0.400	0.050	2.700	
Meats:					
Beef	0.150	0 250	6 500	1 100	0.400
Pork (loin)	1 500	0 200	9 200	1.500	0.600
Poultry (light meat)	0 075	0.060	6 100	0.800	
Poultry (dark meat)	0.100	0 250	7 300	2 000	0 200
Calf's liver	0 400	3.200	20 000	5 200	
Pork liver	0 400	2 700	22 000	5 400	
Milk (whole, fluid)	0.045	0 200	0 070	0.300	0 200
Oatmeal	0.800	0 160	1 130	1.300	0 250
Oranges	0.070	0 030	0 220	0 070	
Peas (fresh)	0.300	0 190	0 750	0.600	
Peanuts	0.800	0 300	13.000	3.400	
Potatoes	0.125	0 060	1.160	0 400	0.160
Spinach	0.075	0 250	0 720	0.200	
Tomatoes	0.050	0.050	0 580	0.075	
Turnips	0.040	0.060	...	0 250	
Yeast (brewers' dry)	12.000	4 000	40 000	20.000	5 500
Whole wheat	0.450	0.120	5.900	1 300	0.460

While the substance producing folic acid from urine, grass and liver has not been isolated, there is growing opinion that it may be related to this xanthopterin, a compound related to uric acid.



Xanthopterin.

* Several claims for its isolation have been made. (See appendix, p. 568).

Xanthopterin is one of a number of pigments, known in general as *pterins*, which were first observed in the wings of butterflies. Uropterin, a very closely related pigment (probably identical with xanthopterin), has been isolated from human urine.*

The vitamin B content of some foods is given in Table 30.

ASCORBIC ACID

Ascorbic acid, known as vitamin C and *cevitamic acid*, is the vitamin the absence of which gives rise to scurvy. This disease, so common among sailors at one time, is characterized by a tendency to

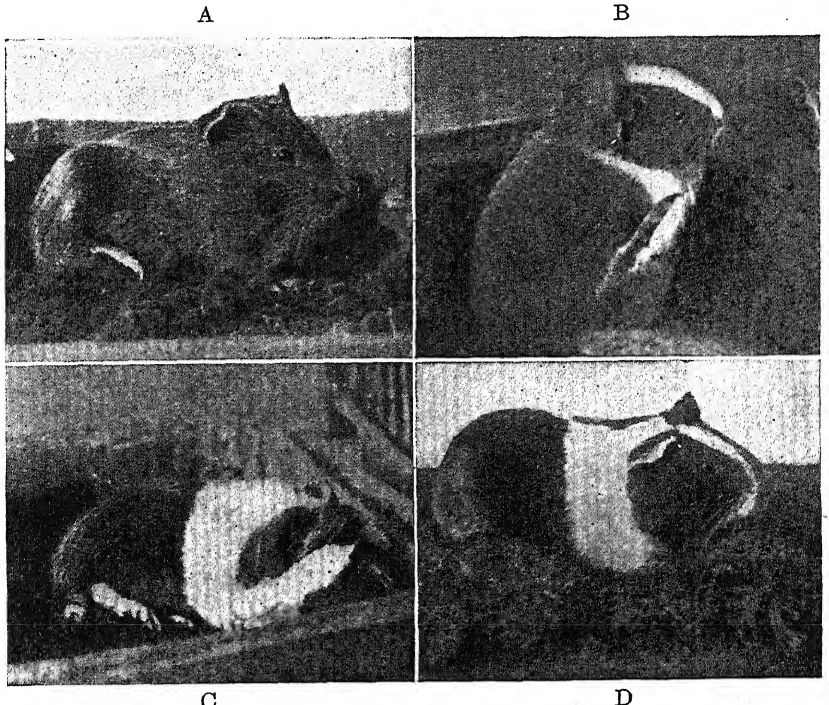


Fig. 50.—The effects of scurvy in guinea-pigs. The joints are inflamed, which may cause the animal to lift his feet off the ground (A and B) to avoid pressing on the painful limb. The attitude shown in (C) has been described as the face-ache position. (D) shows a normal healthy guinea-pig for comparison. (E. M. Delf, *Biochem. J.*)

bleeding, with, among other things, pathological changes in the teeth and gums. In guinea-pigs, in which experimental scurvy can be induced by a diet lacking in "greens," the joints become enlarged and painful (Fig. 50).

Source.—It has been known for some time that fresh fruits and vegetables constitute excellent antiscorbutic sources. Dried cereals and legumes contain practically no vitamin C. Dry seeds, in general, are

* Two additional water-soluble vitamins, B_{10} and B_{11}, have been postulated for chick requirements. These are said to be different from folic acid.

devoid of the vitamin, but as a result of sprouting (by moistening and warming the seeds) the vitamin appears.

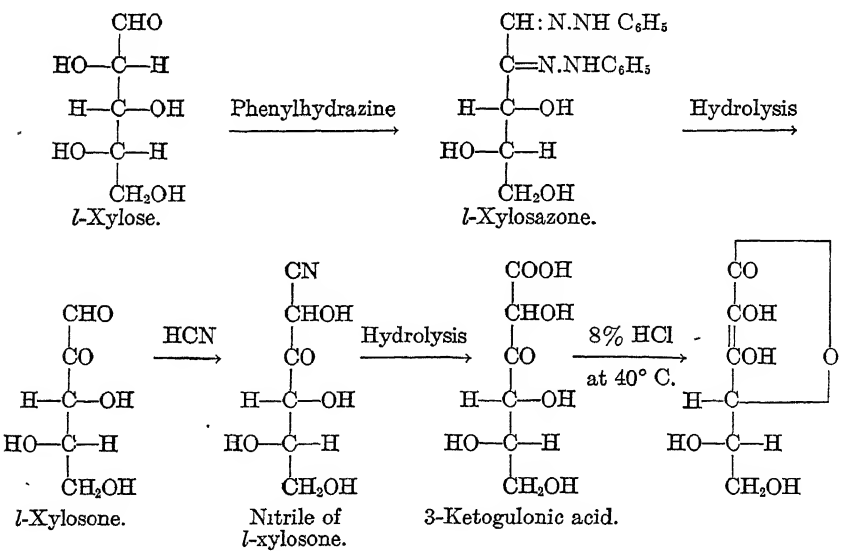
Table 31 gives the food sources of ascorbic acid.

TABLE 31.—FOOD SOURCES OF ASCORBIC ACID. [*Bull. Lederle Labs.* (1942)]

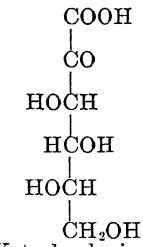
Fair	Good.	Excellent.
Celery	Beef liver	Orange
Asparagus	Papaya	Orange juice
Potatoes	Papaya juice	Grapefruit
Green beans	Cauliflower	Grapefruit juice
Green peas	Cabbage	Lemon juice
Rhubarb	Tomatoes	Broccoli
Bananas	Turnip	Beet greens
	Apples	Strawberries
	Cantaloupe	Horseradish
	Grapes	Kohlrabi
	Cranberry	Peppers—
	Pumpkin	green or red
	Radish	

NOTE: While raw cabbage is a very rich source, sauerkraut is practically lacking in C. Asparagus, another rich source, loses practically all C when cooked.

Chemistry.—Ascorbic acid was isolated by Szent-Györgyi and by King, and its chemistry and synthesis we owe primarily to Haworth and Hirst and to Reichstein. The pure substance has the formula $C_6H_8O_6$, and it is soluble in water and alcohol but practically insoluble in solvents for fats. It is very easily oxidized. One synthesis, as developed by Haworth and his co-workers, started with xylose:

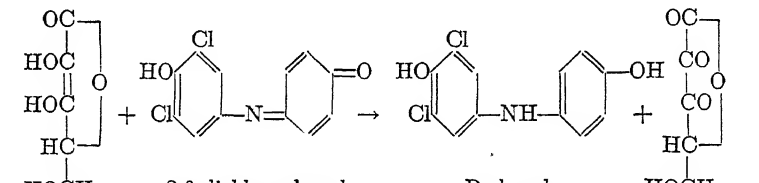


A good commercial method starts with glucose. The glucose is reduced to sorbitol (p. 19) and the latter is oxidized (by selective fermentation) to the keto sugar *l*-sorbose. Oxidation of the sorbose converts it to 2-ketogulonic acid, and in an acid medium the latter lactonizes into ascorbic acid:



2-Keto-*l*-gulonic acid.

Assay.—There are both chemical and biological methods of determining ascorbic acid. One chemical method is based on the reduction of an indophenol dye, 2,6-dichlorophenolindophenol, in acid solution:



Vitamin C

2,6-dichlorophenol-indophenol (blue in alkali; red in acid)

Reduced indicator (colorless).

Dehydroascorbic acid.

The vitamin is first extracted from the tissue by a mixture of acetic and metaphosphoric acids.

Several micro methods adapted for the determination of ascorbic acid in blood have been suggested. One of these methods, in which no more than 0.2 ml. of blood is used, substitutes methylene blue for the 2,6-dichlorophenolindophenol, since the former has a higher color value.

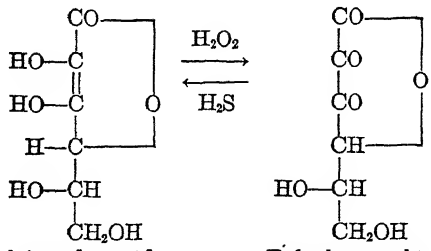
Another method—applied to blood and urine—measures the color developed upon the addition of 85 per cent sulfuric acid to the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid.

In the biological assay, guinea-pigs are used.* The scorbutic diet consists of ground whole oats, heated skim milk powder, butter fat, and table salt; yeast and cod liver oil are sometimes added. Growth stops in about two weeks, the joints become tender, the paws hemorrhagic, and the animal dies in about four weeks. The addition of an extract containing vitamin C to the animal during the scurvy symptoms will bring recovery. The minimum daily dose necessary for the prevention of scurvy is from 10 to 12 International units (one such unit being 0.05 mg. of crystalline *l*-ascorbic acid).

Stability.—Ascorbic acid is among the more unstable of the vitamins. However, care in handling, storing, and preserving foodstuffs has minimized losses. For example, it has been shown that orange juice retains well over 90 per cent of its vitamin C after standing for twenty-four hours in a loosely stoppered flask in a refrigerator.

* Higher plants, and all animals except guinea-pigs, man and other primates, have the ability to synthesize ascorbic acid. The rat, for example, is apparently immune to scurvy.

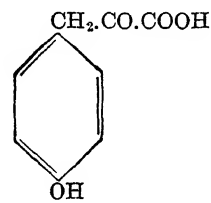
Function.—It is believed that ascorbic acid plays a rôle in biological oxidations, due to the ease with which it is oxidized and reduced:



l-Ascorbic acid. Dehydroascorbic acid.

An ascorbic acid oxidase, present in cabbage, cucumbers, adrenal cortex, etc., catalyzes ascorbic acid oxidations.

The interesting observation has been made that in conditions arising from a deficiency of ascorbic acid—and this applies to infants as well as guinea pigs—there appears in the urine *p*-hydroxyphenylpyruvic acid



which suggests a disturbance in the normal metabolism of phenylalanine and tyrosine (see p. 363).

Requirements.—For daily allowances of ascorbic acid, see Table 13, p. 111.

Such results are arrived at by studying the daily excretion of the acid and by measuring the content of the vitamin in the blood. Using the latter method, and employing as subject a fasting individual, it may be said that, as a general rule, a value of 1.5 to 2 mg. per 100 ml. of blood would indicate satisfactory reserves of the vitamin.

The report of the Council on Pharmacy and Chemistry* of the American Medical Association on vitamin C is as follows:

In planning diet for infants who do not receive breast milk, and for small children, it is generally advisable to make special provision for a source of ascorbic acid, such as orange juice because (a) the concentration of ascorbic acid in fresh cow's milk is only about one-fourth of the concentration in mother's milk, and (b) the vitamin in most foods is very sensitive to destruction by oxidation.

Ascorbic acid is acceptable for the correction and prevention of scurvy. Definite claims for the therapeutic value of ascorbic acid should be permitted only in relation to scurvy until further clinical or experimental evidence has substantiated its usefulness in other states.

It may be permissible under certain conditions to refer to the therapeutic

* *New and Nonofficial Remedies*, 1944.

value of ascorbic acid in early and latent scurvy. Convincing clinical evidence has established that this state does occur. It would be well to emphasize the fact that the diagnosis rests, however, on the basis of roentgenologic evidences in the long bones, the blood level, and possibly failure to excrete an optimum amount of ascorbic acid in the urine.

Dental caries, pyorrhea, certain gum infections, anorexia, anemia, undernutrition and infection alone are not in themselves sufficient indications of ascorbic acid deficiency but according to experimental and clinical investigation may be concomitant signs of ascorbic acid deficiency. Therefore, it is permissible to accept the claim for the therapeutic value of ascorbic acid in these symptomatic conditions *only when* it is definitely stated that they are the consequences of a deficiency or suboptimal amount of ascorbic acid or when there is a pathologic interference with assimilation of the amount necessary for the preservation of health.

Because ascorbic acid is a dietary essential its administration in concentrated form is of value in conditions where difficulty is encountered in introducing it orally or in utilizing ordinary foods in the usual way. Ascorbic acid is accepted as

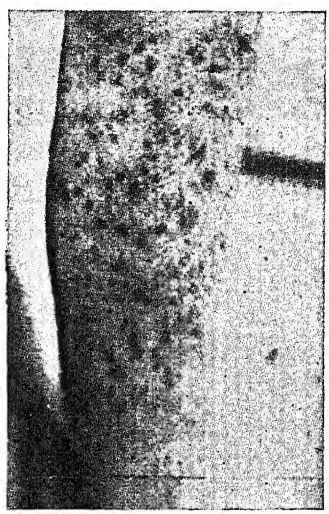


Fig. 51.—Part of the right leg of a patient aged fifty-eight suffering from lack of Vitamin P. A symptom of this is an undue fragility of the capillary walls, with the result that bleeding into the tissues under the skin occurs. (*Monthly Science News*, March, 1943, British Information Service.)

an essential dietary constituent in infant feeding but it should not be accepted for use in the treatment of diseases except according to the conditions mentioned above. . . .

An optimum amount of ascorbic acid should be supplied at all ages for its therapeutic value in preventing the development of acute or latent scurvy.

Advertising of ascorbic acid for such symptoms as failure to gain in weight or stoppage of growth, anorexia, anemia, infections, symptoms referable to the central nervous system or hemorrhagic conditions cannot be accepted unless it is definitely stated that the symptoms are referable to a demonstrable deficiency of ascorbic acid.

Ascorbic acid is easily decomposed in the presence of certain other substances; therefore, care should be exercised against administering it (or orange juice) in mixtures, or by any procedure which renders it ineffective.

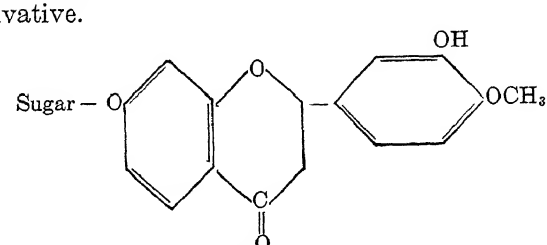
Glucoascorbic Acid and Scurvy.—The feeding of glucoascorbic acid, a substance structurally related to ascorbic acid, to mice led to a scurvy-like condition in the animal (Woolley). This is in accord with a theory of chemotherapy first developed in connection with the relation of *p*-aminobenzoic acid to sulfanilamide (p. 169. See also Chap. 14).

VITAMIN P (CITRIN)

Though some uncertainty still exists regarding this vitamin, the activity in this field warrants brief mention at this point.

Szent-Györgyi suggested in 1936 that, aside from ascorbic acid, extracts of Hungarian red pepper and lemon juice contain a substance which controls certain types of hemorrhages. The name "vitamin P" was suggested for the substance because, it is claimed, it controls vascular permeability. Extracts have been prepared from lemons; hence also the name "citrin." Work on the chemistry of the material led to the conclusion that it was a mixture of two flavones in the form of their glucosides.

It has been claimed that vitamin P is present as one group in an enzyme isolated from lemon peel. By reduction and ring closure the prosthetic grouping in the enzyme is converted to hesperidin, a flavone derivative.



Hesperidin

Hesperidin decreases capillary fragility and prevents hemorrhages (Fig. 51).

VITAMIN D

"Vitamin D" represents more than one substance which deals with the proper utilization of calcium and phosphorus. It is not only important in bone formation but also in tooth formation.

Calciferol, or vitamin D_2, is one crystalline form obtained by the ultraviolet irradiation of ergosterol. Vitamin D_3, another crystalline modification, is obtained by the irradiation of 7-dehydrocholesterol.

The irradiation of ergosterol giving calciferol (the oil solution is known as *viosterol*) and the activated 7-dehydrocholesterol are the only two among ten forms of vitamin D which are of practical importance.

The 7-dehydrocholesterol is present in animal fats and is "activated" naturally when skin, feathers or furs of animals are exposed to sunlight or other sources of ultraviolet rays.

In the absence of this vitamin, or in its presence in insufficient amounts, rickets in varying degree of severity develops. In the child (Fig. 52), the disease is associated with bowlegs, knock knees, swollen joints, etc. The growing parts of the bone—particularly the ends of the long bones of arms and legs—are affected. An *x*-ray examination makes diagnosis relatively simple. In experimental animals (Fig. 53) an analysis of the bone ash is also of diagnostic significance. In rickets

the ends of the bones show incomplete calcification; as healing advances the material becomes more dense (Figs. 54 and 55). Two other diagnostic tests of value are the decrease of the phosphorus content of the blood in rickets* (normal amounts are obtained upon healing),

Fig. 52.—Children six years of age showing severe rachitic deformities compared with normally grown child (center) of the same age. (Vienna, 1920. Amerikanische Kinderheilstätte.)

and an increase of the enzyme phosphatase in the blood during the disease (with a decrease of the phosphatase upon healing).

* As a rule, it is the inorganic phosphorus, rather than the calcium, which shows low values. The normal phosphorus values for an infant are 4–6 mg. per 100 ml. of blood. Values below 3.5 mg. are of diagnostic significance. Sometimes, however, there is a decreased concentration of calcium, and sometimes a decreased concentration of both elements. In any case, we may regard the situation as involving a lowering of the solubility product—a factor which regulates the precipitation of calcium phosphate from the blood into the cartilage and bone.

When the concentrations of calcium and phosphate ions are decreased, and therefore the product of their concentration is decreased below the solubility product of the bone salt, "lime salt deposition in bone and cartilage becomes irregular and, if the value is low enough, deposition stops altogether. The failure in lime salt deposition is responsible for the weakness of the bones. This, in turn, results in the development of the well-known deformities of the disease and at the same time is the cause of almost, if not all, the histological changes. The first demonstrative pathological change in rickets is the failure of lime salt deposition in the proliferative cartilage of the epiphysis and in newly forming bone" (Park).

From the chemical standpoint, the problem of calcium and phosphorus precipitation is, in reality, a complicated one. The ions involved are not only calcium and phosphate but carbonate as well. The salt precipitated probably has the composition $n Ca_3(PO_4)_2 . CaCO_3$, where n may stand for 2 or 3. The salt may be regarded as a solid solution of $CaCO_3$ in $Ca_3(PO_4)_2$.

A large part of the serum calcium is combined with protein. Out of a total of 10 mg. of calcium in 100 cc. of serum, about 4.5 mg. is ionized.

Phosphatase can decompose organic phosphorus compounds—hexose phosphate, for example—into inorganic phosphate. There are several phosphatases. The particular one of importance here is known as the "alkaline phosphatase" with an optimum *p*H action of about 9. This one, in contradistinction to the "acid phosphatase" (about *p*H 5), is widely distributed in ossifying cartilage, bone, kidneys, intestinal mucosa, liver. It is found, in relatively smaller amounts, in blood serum.

In rickets of infancy and early childhood, the serum alkaline phosphatase may be high. From a normal of 5 to 15 units per 100 cc. of blood, it may increase to 20–30 units in mild cases, 40–60 units in moderately severe cases, and up to 190 units in very severe cases.*

In addition to rickets, vitamin D has been used to treat infantile tetany, characterized by a low calcium content of the blood (in contrast to the usual low phosphorus content in rickets). It is true that the parathyroids (p. 483) play an important rôle in the metabolism of calcium, but it would seem that both the hormone and vitamin D are involved.

Treatment with vitamin D has also been used in cases of osteomalacia, an adult disease characterized by the softening of the bones.

Function.—It is believed by many that the primary function of vitamin D is to regulate the absorption and utilization of calcium and phosphorus. As evidence of this, it is pointed out that in rickets a relatively large quantity of calcium and phosphorus is lost in the feces.

That increased absorption alone is not sufficient to account for the function of the vitamin is brought out by the work of Greenberg. Using radiophosphorus (P^{32}) this author studied the influence of vitamin D on the phosphorus metabolism of rachitic rats. The increase in the absorption of phosphate (administered by stomach tube) was from 10 to 15 per cent. However, the more striking changes occurred in the phosphorus fractions of the bone. The lipoid phosphorus (alcohol-ether soluble P) was not altered, but the labeled phosphorus of the inorganic fraction increased by 40 per cent.

The absorption of vitamin D, like the absorption of fats and fat-soluble foods in general, is largely dependent upon the presence of bile salts.

The liver is the chief storage place for vitamin D, though smaller amounts are found in other organs (skin, brain, lungs, spleen and bones).

Vitamin D, suggests Greenberg, may act to aid the conversion of organic to inorganic phosphorus in bone. The vitamin exerts an influence on the process of mineralization in bone.

Assay.—The study of rickets was greatly facilitated by devising diets which readily produced rickets in rats and in chicks (Figs. 54; 55). Usually, such diets contain relatively large quantities of calcium, little phosphorus, and no vitamin D. For example, the composition of one

* The estimation is based upon the amount of phosphorus liberated as PO_4 ions by a given amount of serum incubated with sodium β-glycerophosphate and buffered at *p*H 8.6. The "unit" represents the number of mg. of phosphorus which 100 cc. of the serum can liberate in one hour as phosphate ions.

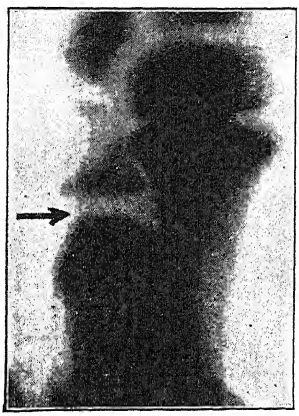
such diet for the rat is whole yellow corn, 76 per cent; ground gluten, 20 per cent; calcium carbonate, 3 per cent; sodium chloride, 1 per cent. Here the ratio of Ca:P is nearer to 4.5:1 than to the normal ratio, which is 1.2:1. At the end of some three weeks on this diet, the uncalcified zone in the epiphyseal section of the long bone can be detected by means of *x*-rays. Furthermore, the phosphorus in the blood drops from a normal of 6 to 8 mg. per 100 cc. to 2 to 3 mg., and the bone ash, from

Fig. 53.—Two puppies from the same litter, both given an identical diet except that the one below was given the *addition of a trace of vitamin D concentrate*, and remained normal; while the one above had none and so developed rickets (Steenbock).

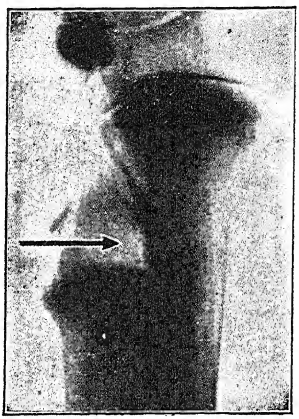
a normal of 50 to 60 per cent to 25 to 35 per cent. As a rule, the calcium content of the blood does not change materially. In fact, the common forms of rickets in children show little change in calcium but a very definite drop is phosphorus.

A spectrophotometric method which has been proposed is based on the reaction between the vitamin and a chloroform solution of antimony trichloride and acetyl chloride, giving rise to a yellowish pink color. The maximum absorption curve of the reaction product is at 500 mμ.

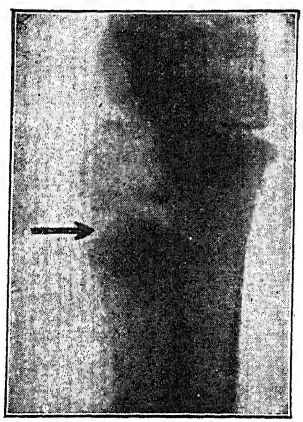
Recovery from Rickets.—Healing may be accomplished in one of several ways. One of the oldest remedies is to feed cod liver oil, one of the few natural foods rich in vitamin D.* Another is to expose the child or animal to the sun's rays. Here the ultraviolet rays convert the provitamin in the skin (probably 7-dehydrocholesterol, p. 186) into



8½ weeks: Wide cartilaginous zone, normal for young animal.



17 weeks: Extremely wide cartilaginous zone; note marked irregularity of end of shaft.



8½ weeks: Wide cartilaginous zone, normal for young animals.

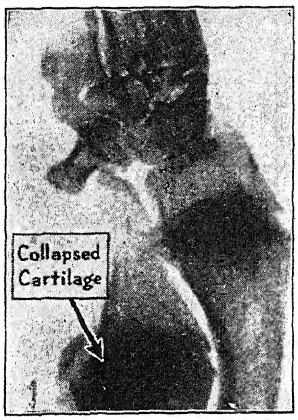
17 weeks: Narrow cartilage and well-defined head of shaft—normal for this age.

Fig. 54.—Upper, both x-ray and external signs of rickets. Lower, normal animal for purposes of comparison. (Fleischmann Laboratories, Standard Brands Incorporated.)

vitamin D, which then exerts its influence on mineral metabolism. The antirachitic region of the spectrum is from 256 mμ to 313 mμ, and the shortest solar radiation is 290 mμ; so that relatively little benefit

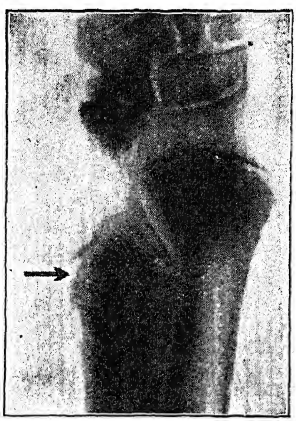
* "When vitamin D is given to rachitic animals . . . the first histological evidence of repair is the presence of degenerated cartilage cells. The effect is visible at the end of twenty-four hours and is accompanied by extensive vascular penetration within forty-eight hours. The penetration of blood vessels permits the deposition of the bone-forming salts. There is thus produced the so-called 'line-test' for healing" (Shohl).

is obtained except on very clear days and preferably "in the open spaces." This has led to the extensive use of the carbon and mercury arcs, which give off ultraviolet rays, and to the employment of quartz glass in the place of ordinary window glass, because the former, unlike the latter, is not opaque to such rays. The discovery, both by Steenbock and by Hess, that foods devoid of vitamin D can be made anti-



20 weeks: Cartilage crushed under animal's weight; note mushrooming of head of shaft around base of epiphysis.

28 weeks: Deformity of legs resulting from severe rickets.



22 weeks: Still narrower zone of cartilage—normal appearance for this age.

28 weeks: Normal straight legs.

Fig. 54.—(*Continued.*)

rachitic by exposing them to rays from the carbon or mercury arc has proved important in developing preventive measures against the disease.

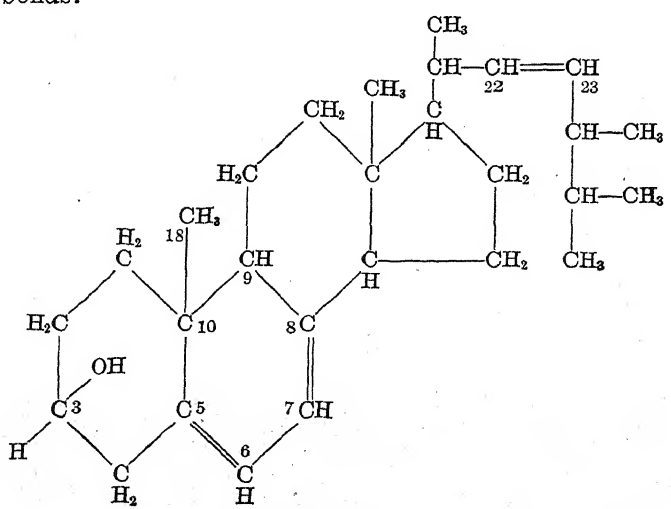
Chemistry.—The attempts to isolate the antirachitic vitamin have led to brilliant results. The vitamin is found in the unsaponifiable fraction of cod liver oil. For a time it was supposed that the irradiation of cholesterol itself (found in the unsaponifiable fraction) produced vitamin D, but a rigorous purification of this sterol, involving its con-

version into the dibromide and reconversion into the sterol, led to negative results. Windaus finally traced the mother substance to a closely associated sterol, ergosterol, originally found in ergot (hence its name).

The irradiation of ergosterol produced an antirachitic substance which was finally isolated and named "calciferol"* by an English group of investigators and vitamin D_2 by Windaus. The name "viosterol" (an oil solution) has been adopted by the Council of Pharmacy and Chemistry of the American Medical Association.

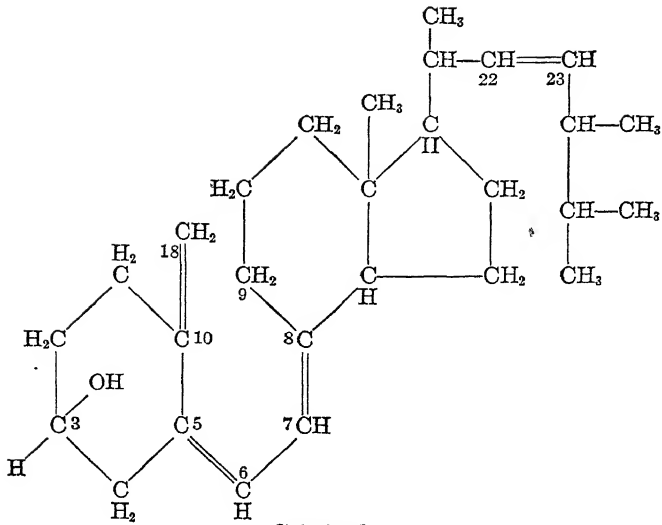
Fig. 55.—Rickets in the chick. The response of chickens and rats is the principal means of determining potency of vitamin D products. In rats healed rickets shows as a line of calcification across the metaphysis of the cut long bone. The photograph shows "leg weakness" which is typical of rickets in chickens. Amount of bone ash is the criterion of calcification in chickens. Day-old chicks are fed a rickets-producing ration. Control birds receive no vitamin D; others are given substances to be tested. After four weeks the chicks are killed; the left tibia is removed, extracted, and dried to constant weight. Bones of each group are ashed collectively, percentage of ash is determined in terms of dry bone weight. (*Therapeutic Notes*, by courtesy of Parke, Davis & Co.)

Ergosterol is mainly distinguished from cholesterol by having three double bonds:



Ergosterol.

* It is also known as "drisdol."



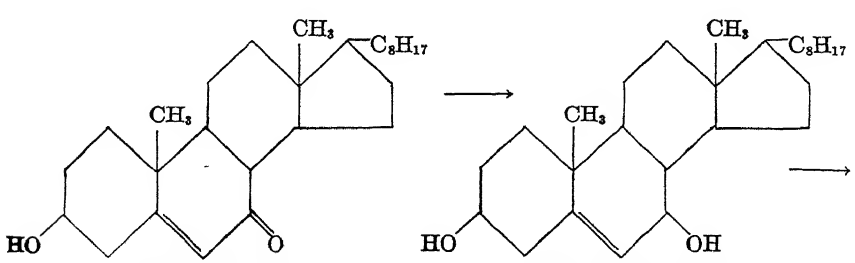
Calciferol.

Its conversion to a vitamin D is due to the opening up of the second ring and the introduction of a fourth double bond.

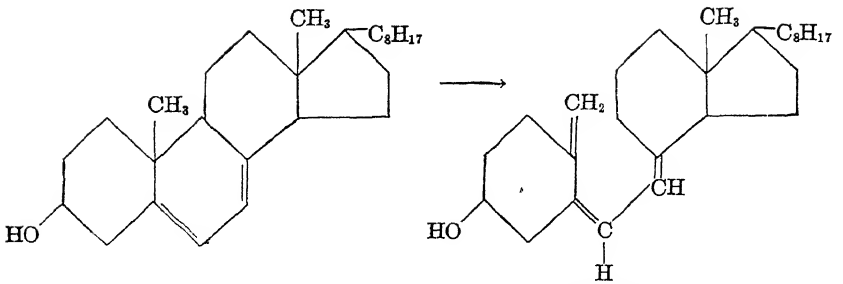
The structures of the antirachitic substances are characterized by the opening of ring B, and the replacement of a methyl group at position 10 by a methylene group, giving rise to three conjugated double bonds.

Actually, the irradiation of ergosterol produces a series of substances only one of which, calciferol, is antirachitic: ergosterol → lumisterol → tachysterol → calciferol → toxisterol → suprasterols (I and II). What was originally called vitamin D has proved to be a mixture of lumisterol and calciferol; hence the renaming of calciferol as vitamin D_2.

Several D Vitamins.—That irradiated ergosterol (calciferol) and the antirachitic vitamin in cod liver oil are not the same was made probable by the observation that calciferol was not as effective in curing chicks as an equivalent quantity of vitamin D in cod liver oil (based on rat assay). But the suspicion that there were several provitamins and several vitamins D was considerably strengthened by the brilliant research of Windaus. Starting with 7-ketocholesterol, ob-



7-Ketocholesterol. 7-Hydroxycholesterol.



7-Dehydrocholesterol. Vitamin D_3.

tained by oxidizing cholesterol acetate with chromic acid, Windaus reduced it to 7-hydroxycholesterol. The dibenzoate was heated in vacuo at 200° C., whereby a molecule of benzoic acid was split off and another double bond introduced in position 7:8. This substance is 7-dehydrocholesterol, and its double bond arrangement in the second ring corresponds to that of ergosterol. The ultraviolet absorption spectra of the two are the same. The irradiation of 7-dehydrocholesterol gave rise to an antirachitic substance which was isolated and found to be more active than calciferol in tests on chicks. This antirachitic substance is called vitamin D_3. "It seems probable," write Schoenheimer and Evans, "that the provitamin and vitamin D in the organs of higher animals are derivatives of cholesterol rather than ergosterol."

7-Dehydrocholesterol and vitamin D_3 are substances of animal origin exclusively. Ergosterol and calciferol, though essentially vegetable products, are also found in the animal organism and originate in the animal's food.*

7-Dehydrocholesterol, which may be regarded as the naturally occurring provitamin D, has been isolated from fish liver oils.

Wintersteiner has shown that oxidation (by air) of cholesterol gives rise to 7-ketocholesterol (p. 40). This suggests a possible intermediate in the formation of vitamin D (D_3) in the animal body.

Distribution.—Vitamin D is not very liberally distributed. It is found in the flesh of all fat fish. For example, cod liver oil and the livers of the halibut, tuna, and sword fish, etc. Little of the vitamin is found in natural foods. Much of the vitamin D we need is obtained on exposure to the sun (by irradiation of the provitamin in the skin).

On a comparative scale, next to fish livers, the richest source is egg yolk, followed by butter fat. Milk is quite poor in the antirachitic factor, though it becomes a simple procedure to enrich it with vitamin D. This may be done by the addition of vitamin D concentrates, by irradiation of the milk, or by feeding cows with foods rich in the factor. "Milk fortified with vitamin D," to be accepted by the Council of

* The D "provitamins," such as ergosterol and 7-dehydrocholesterol, show ultraviolet absorption characteristics of conjugated dienes, with the double bonds in the same ring. In the corresponding vitamins, the absorption spectra typify conjugated trienes

Foods of the American Medical Association must contain from 135 to 400 U S.P. units per quart of milk.*

Requirements.—See Table 13, p. 111.

Vitamin D does not decrease the requirements of calcium and phosphorus; the vitamin cannot produce good retention of these elements when they are present in insufficient amounts.

A number of clinical reports suggest that the administration of vitamin D preparations in unusually large dosages is not unaccompanied by danger.

In the opinion of the Council on Pharmacy and Chemistry† of the American Medical Association, the allowable claims for vitamin D are the following:

Vitamin D is recognized as a specific in the treatment of infantile rickets, spasmophilia (infantile tetany) and osteomalacia, diseases which are manifestations of abnormal calcium and phosphorus metabolism. Vitamin D is valuable in the prevention as well as in the curative treatment of these diseases. Complications such as renal insufficiency or glandular malfunction may preclude normal response to vitamin D therapy. During acute infections, especially of the gastrointestinal tract, vitamin D may prove ineffective because poorly absorbed.

Direct exposure of the skin to ultraviolet light from the sun or from artificial sources results in the formation of vitamin D within the organism, but the Council cannot recognize statements or implications that vitamin D has all the beneficial effects of exposure to sunshine.

There is clinical evidence to justify the statement that vitamin D plays an important role in tooth formation. Its other values in relationship to teeth are still subject to investigation.

Animal experimentation has shown that correction of an inadequate intake of vitamin D results in the more economical utilization of calcium and phosphorus and also that the undesirable effects of improper ratios of calcium and phosphorus in the diet can largely be overcome by normal intake of vitamin D. The importance of these observations in their application to man is not entirely apparent because of the lack of adequate clinical evidence showing the availability of different forms of calcium and phosphorus, but it may be stated that vitamin D has a favorable influence on calcium and phosphorus metabolism.

Because of its effect upon the level of serum calcium, vitamin D has been used in correcting the hypocalcemia of parathyroid tetany (p. 483). Satisfactory effects may be obtained with sufficient doses either of vitamin D_2 (calciferol) or of dihydrotachysterol, a derivative of one of the products resulting from the irradiation of ergosterol. When vitamin D preparations are employed for the correction of hypocalcemia, patients must be under constant observation since the elevation of serum calcium above normal levels may be accompanied by serious or even fatal effects.

Clinical evidence does not warrant the claim that massive doses of vitamin D are of benefit in chronic arthritis, in allergic disorders, or in psoriasis. If representations are made for use of massive doses of vitamin D in the treatment of refractory rickets they must be accompanied by adequate precautions with respect to the danger of toxic effects and how they can be avoided as indicated in the paragraph immediately preceding the allowable claims for vitamin D.

VITAMIN E (ALPHA-TOCOPHEROL)

Until 1922 it was supposed that a synthetic diet for rats could be used which would cause normal growth and reproduction. Such a diet included casein, starch or sucrose, lard, salts, cod liver oil, and yeast. Evans and Bishop, and then Sure, showed that while this diet allowed

* Mellanby showed sometime ago that the addition of certain cereals to a diet low in calcium leads to rickets in animals. The substance responsible has been traced to phytic acid (inositol hexaphosphoric acid) or to phytin itself (p. 170). It is believed that phytic acid precipitates calcium (or converts it into an unionized form) so that it cannot be absorbed through the intestine.

† *New and Nonofficial Remedies*, 1944.

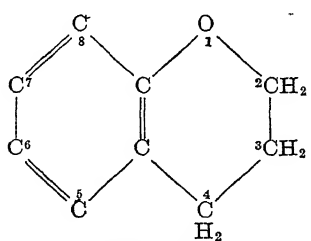
for apparently normal growth the reproductive process was very definitely interfered with. Only by incorporating in this diet small quantities of certain natural foods—cereal grains, green leaves, legumes, nuts, and particularly the oil from wheat germ—could reproductive disturbances be avoided. The necessary factor is known as vitamin E. In the absence of vitamin E the germinal epithelium of the testes of rats is destroyed. In the female rat, ovulation and fertilization take place, but also there is death and resorption of the fetus. This situation can be repaired by incorporating vitamin E in the diet.

Properties.—Vitamin E, like vitamins A and D, is soluble in fat solvents and insoluble in water. It is extremely resistant to heat but readily oxidized. Like vitamins A and D, it is found in the nonsaponifiable fraction of fats and oils. Out of the nonsaponifiable fraction of wheat-germ oil, Emerson and Evans succeeded in isolating and crystallizing two substances, to which the names α-tocopherol and β-tocopherol were given (*tokos* = childbirth; *phero* = to bear; *ol* = alcohol). The α form was biologically much the more active of the two: as little as from 1 to 3 mg. doses proved effective. Somewhat later the α modification was also isolated from cottonseed oil. A γ-modification has also been obtained.

Since the α-form is biologically the most potent and is now readily available as a synthetic product, this modification is the one that is almost invariably used. In general, when we speak of vitamin E we mean α-tocopherol.

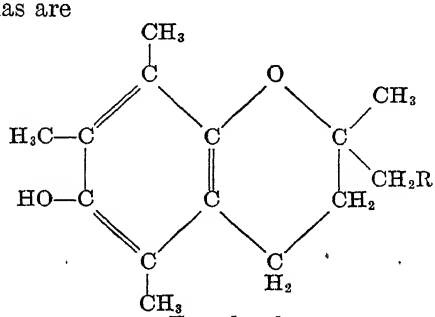
Source.—The vitamin is found in green lettuce leaves, whole cereals (oats and wheat), beef liver, egg yolk, wheat embryo, etc. Wheat germ oil is the richest source.

Chemical Structure.—The three tocopherols are derivatives of chromane:

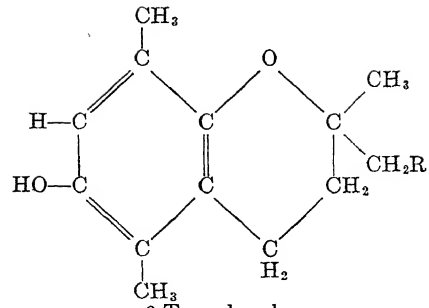


Chromane.

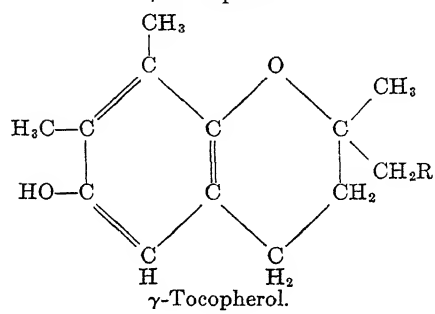
and their formulas are



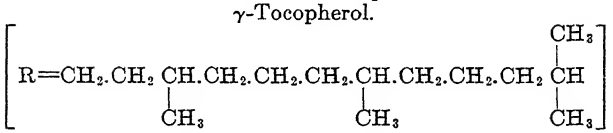
α-Tocopherol.



β-Tocopherol



γ-Tocopherol.



It will be seen that the structural differences of these three compounds are confined to the benzene ring. The α-modification has three methyl groups in positions 5, 7, 8; the β-, two methyl groups in positions 5, 8; and the γ-, two methyl groups in positions 7, 8.

The tocopherols have not only been isolated but also synthesized.

Structure and Biological Activity.—The methyl groups in the benzene ring are important. The β- and γ-tocopherols, with two methyl groups in the ring, are less active than α-tocopherol, with three methyl groups. Karrer has shown that the monomethyl derivative (which he synthesized) is quite inactive.

On the other hand, several phenols and quinones, but distantly related to vitamin E in chemical structure, show varying biological activities.

Physiological Functions.—These functions seem many-sided. Aside from the influence that vitamin E has on the reproductive system, it has also been connected with the physiology of the muscular and vascular systems.

Vitamin E can be easily oxidized and destroyed by treatment with ferric chloride. Goettsch and Pappenheimer showed that rabbits and guinea-pigs kept on a vitamin E-free diet (the vitamin in the food had been destroyed by ferric chloride) developed a dystrophy of the voluntary muscles (atrophy of the muscles). The deficiency disease can be cured by α-tocopherol, provided there is enough of the vitamin B complex.

It is possible to produce muscle dystrophy in rats and dogs, as well as in guinea-pigs and rabbits; here again the cure lies in adding α-tocopherol to the diet.

A number of herbiverous animals develop muscular dystrophy by the addition of cod liver oil to their diet. It is believed that this is due to the unsaturated fatty acids in the oil which destroy the vitamin E in the diet or in the digestive tract.*

Assay.—The vitamin activity is expressed as mg. of material, fed in a single dose, which is required to cure the sterility and produce litters in 50 per cent of the animals used.

A vitamin E-deficient diet, used by Evans and his coworkers is the following:

Casein, commercial, precipitated with HCl	27
Cornstarch (cooked)	35
Salts (McCollum 185)	4
Lard	22
Cod liver oil (Squibb's)	2
Brewer's yeast	10

The mixed diet without the cod liver oil is allowed to stand for 2 weeks at room temperature to permit the rancid substances of the lard to destroy incipient traces of vitamin E. The cod liver oil is added just before feeding

It has been proposed that the international unit of vitamin E be defined as the specific activity of 1 mg. of α-tocopherol acetate (which is the average amount, administered by mouth, which prevents resorption gestation in rats deprived of vitamin E).

Besides the biological assay, other methods have been suggested: measurement of the absorption spectrum (294 mμ); potentiometric titration with gold chloride; oxidation of the tocopherols with ferric chloride, the conversion of the resulting ferrous ion to a red complex with α,α′-dipyridyl and measuring the red color;† oxidation of the tocopherols with nitric acid to form red-colored compounds, the color of which can be measured.

The chemical methods make no distinction between the three tocopherols. Since biologically the α-tocopherol is the most potent of the three, the rat assay, which does distinguish biological activity, still has to be used.

Vitamin E Applied to Humans.—It would have been surprising if this so-called "antisterility" vitamin had not given rise to hopes which, so far, have not been realized. Conflicting results have been obtained by its use in "threatened abortion" and in muscular dystrophy; and it seems to have little or no value when applied to cases of sterility.

VITAMIN K

Dam and later Almquist described a hemorrhagic disease in chickens due to a food deficiency. The disease is associated with a decrease

* Incidentally, this is a two-sided reaction. The rancidity of fats can be prevented in a measure by the addition of vitamin E.

† Mild oxidation—with gold chloride or ferric chloride—breaks the oxygen ring in tocopherol and converts the benzene ring into a quinone.

in the amount of prothrombin (p. 267) in the blood. The factor missing from such a diet, which is associated with the fat-soluble fraction, has been given the name *vitamin K* (after Dam, who named it "Koagulations vitamin").

Vitamin K and the Clotting Process.—The theory of the blood clotting process as at present understood (p. 267) is that thromboplastin, liberated from wounded tissue cells or from disintegrated blood platelets, together with calcium ions, converts prothrombin (a proenzyme or zymogen) into thrombin (an enzyme); and once the thrombin is formed it converts the fibrinogen of the plasma into insoluble fibrin (blood clot). Vitamin K is necessary for the formation of prothrombin—a process which occurs in the liver.

Vitamin K-Deficient Diet.—As a rule, chicks are used because the disease is easily produced. Vitamin E should be included. To prevent perosis (p. 171), choline and manganese are added. Almquist has shown that for optimal growth glycine, creatine and glucuronic acid should be included. Glycine may be offered in the form of gelatin and glucuronic acid as gum arabic. Yeast supplies the vitamin B complex. Vitamins A and D are given in the form of cod liver oil, and vitamin E as wheat germ oil or pure *dl*-alpha-tocopherol acetate.

Other basic material may be supplied by fish meal (ether extracted) or sardine meal (ether extracted), or polished rice, sucrose, calcium carbonate, sodium chloride, ferric citrate, and copper sulfate.

Since vitamin K is synthesized during putrefaction, chicks must be prevented from soiling their food and water with feces.

The synthesis of vitamin K in the intestinal tract during putrefaction has made it difficult, for example, to produce a vitamin K deficiency in the rat. This has now been overcome by adding to the diet one of the more insoluble "sulfa" drugs, such as sulfaguanidine or succinylsulfathiazole, which most probably prevent bacterial synthesis of the vitamin.

Assay.—One method is to place day-old chicks on a vitamin K-deficient diet. Bleeding is performed at the end of twelve days. If 90 per cent of the 10 or 12 birds selected for the bleeding show a coagulation time of sixty minutes or more, the flock is considered ready for assays. The substance to be tested for vitamin K is added to the diet, and the effect on the reduction of clotting time is noted.

An effective dose is the minimum amount of material which will reduce the clotting time of 60 to 80 per cent of the vitamin K-deficient birds to less than ten minutes. Fieser and Tishler find that 0.3 micrograms of 2-methyl-1,4-naphthoquinone is such an effective dose, and may be considered as one unit.*

Methods other than the biologic assay with chicks have been suggested. One deals with the ultraviolet absorption of the vitamin (or rather vitamins, for there are two of them; p. 193). Another involves the red-brown color produced with sodium ethylate. Still another method employs the catalytic hydrogenation of the quinone to the

* Unfortunately, many units have been proposed and there is, at present, no general agreement.

orresponding hydroquinone and the titration of the latter with ,6-dichlorophenolindophenol (p. 175).

Occurrence.—The green leaf and chlorophyll-containing plant rgans (alfalfa, spinach, etc.) are rich in vitamin K With the exception of tomatoes, fruits contain little. Little is found in cereals and beans, and practically none in carrots and potatoes.

Some bacteria contain considerable quantities of the vitamin. Yeast contains practically none of it.

Among substances tested, spinach, cabbage, kale and cauliflower are good sources.

Vitamin K occurs less abundantly in the animal than in the plant world. Liver may be—but is not always—fairly rich in this vitamin. Milk—whether human or cow's—is a poor source.

The feces are rich in vitamin K.

Function.—Though the part vitamin K plays in blood clotting is undoubtedly important, the fact that the vitamin is so widespread suggests other as yet unknown functions.

The vitamin is powerless in hemophilic conditions (p. 271). Since many believe that in hemophilia the primary disturbance is due to difficulty in forming thromboplastin, and since vitamin K is primarily concerned with the formation of prothrombin, the result is not unexpected.

Just how vitamin K helps in the formation of prothrombin is not known; but we do know that the reaction takes place in the liver.

It has been suggested that vitamin K is changed in the body to phthalic acid, which is considered the real antihemorrhagic substance, but this theory awaits confirmation.

Clinical Application.—Vitamin K has proved valuable in treating cases of obstructive jaundice (where there is an obstruction in the flow of bile) and in a number of liver diseases. In both examples we find deficient quantities of prothrombin in the plasma. This deficiency is explained partly on the ground that when bile is excluded from the intestinal tract, the absorption of fats (and hence fat-soluble substances, like vitamin K) suffers (see Chap. 12); and another partial explanation is that the liver needs vitamin K for the synthesis of prothrombin.

The newborn baby often shows a low prothrombin content. This can be improved by giving the mother the vitamin some time before delivery, or by direct ingestion by the baby.

There are a number of hemorrhagic conditions which do not lend themselves to treatment with vitamin K; and it is by no means a cure-all for all types of hemorrhages. The vitamin, for example, is not effective in hemophilia (p. 271), scurvy (p. 173) or gastric ulcer.

Chemistry.—A surprising amount of work has been accomplished in a comparatively short time. Dam, Almquist, Karrer, Doisy and his coworkers, Fieser, and others have done much to elucidate the problem.

Starting with alfalfa, the fat-soluble vitamin K was isolated as an oil; but when the source was putrefied fish meal, the vitamin was obtained in crystalline form. This immediately suggested two forms of

vitamin K: vitamin K_1, manufactured by the green leaf, and vitamin K_2, formed during the course of putrefaction.

An absorption maximum for vitamin K_1 is at 248 mμ.

With sodium ethylate the vitamin forms a blue color which changes to a reddish-brown.

Ozonolysis of K_1 led to the isolation of a product which could also be obtained from phytol, and another product which was finally identified as 2-methyl-1,4-naphthoquinone-acetic acid The presence of a benzene ring adjoining the quinone part of the molecule was shown by oxidation with chromic acid, yielding, as one of the products, phthalic acid.

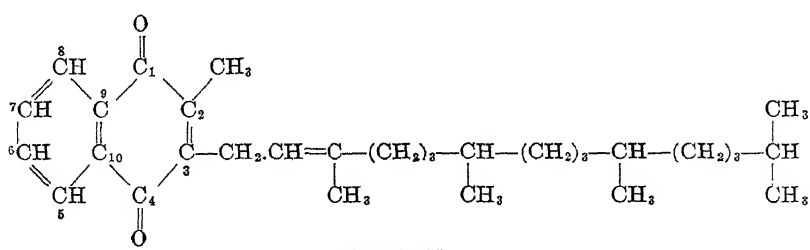
TABLE 32.—COMPARATIVE ACTIVITIES OF THE MORE IMPORTANT ANTIHEMORRHAGIC COMPOUNDS BASED ON RECENT CHICK 5-DAY ASSAYS AND EXPRESSED IN 2-METHYL-1,4-NAPHTHOQUINONE UNITS PER MILLIGRAM. (Almquist, *Physiol. Rev*, **21**, 194)

	Units per milligram
2-methyl-1,4-naphthoquinone (menadione)	1,000
2-methyl-1,4-naphthohydroquinone	930
2-methyl-1,4-naphthohydroquinone diacetate	450
2-methyl-4-amino-1-naphthol hydrochloride	470
2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester (tetra sodium salt + 6 molecules water)	490
2-methyl-3-phytyl-1,4-naphthoquinone (vitamin K_1):	
Natural	300
Natural, dihydro diacetate	100
Synthetic	290
2-methyl-3-(?)-1,4-naphthoquinone (vitamin K_2)	240

Synthesis of vitamin K was accomplished by condensing phytyl bromide with the sodium salt of 2-methyl-1,4-naphthoquinone.

Vitamin K_2 yields the same quinone and also phthalic acid, but the side chain is more unsaturated and is longer.

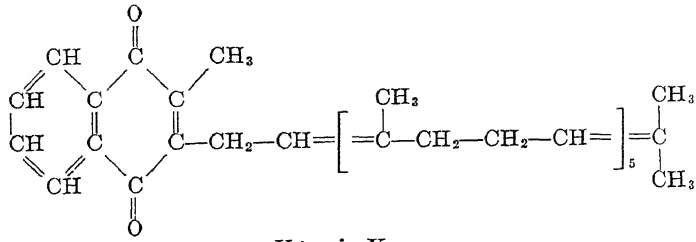
Vitamin K_1 is 2-methyl-3-phytyl-1,4-naphthoquinone.*



Vitamin K_1.

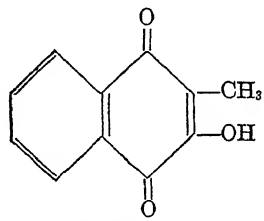
Vitamin K_2 is also a derivative of 2-methyl-1,4-naphthoquinone, but with a side chain at position 3 other than phytyl. The probable formula is

* For the phytol group in chlorophyll, see p. 207.



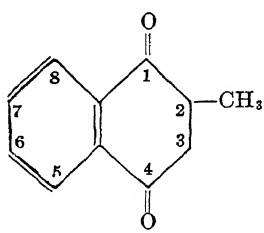
Vitamin K_2.

1,4-Naphthoquinone itself shows vitamin K activity. It is also of interest that phthiocol, isolated by Anderson and associates as the pigment in human tubercle bacilli, and a 1,4-naphthoquinone derivative, shows slight antihemorrhagic properties.



Phthiocol
(2-methyl-3-hydroxy-1,4-naphthoquinone).

However, 2-methyl-1,4-naphthoquinone (known as *menadione*) shows a biological activity which may be somewhat higher than vitamin K_1 itself.*



2-Methyl-1,4-naphthoquinone (Menadione).

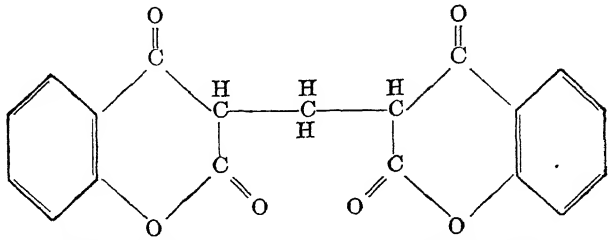
Comparative Activities of Some Anti-hemorrhagic Compounds.—Table 32 points to the fact that menadione is about three times as potent as vitamin K_1 or K_2.

In many cases the activity seems to be associated with the ease with which each compound can be converted to menadione in the body.

Dicumarol.—An interesting *hemorrhagic* agent has been uncovered in "sweet clover disease." This disease in cattle arises from eating improperly cured hay or silage made from common sweet clover. The disease shows itself by a progressive diminution in the clotting power

*Link states that the oral administration of menadione in large quantities to animals (dog, rabbit and rat) induces a *hyper*prothrombinemia, which may persist for several days.

of the blood and resultant hemorrhages which usually become fatal. The hemorrhagic agent has been isolated and identified by Link and co-workers and proves to be



3,3′-methylenebis-(2,4-diketochromane) or 3,3′-methylenebis(4-hydroxycoumarin).

This substance, known also as *Dicumarol*, has also been synthesized.*

This dicumarol acts antagonistically to vitamin K, in that the hypoprothrombinemia induced in the rat (and in man) by dicumarol can be partially nullified by vitamin K.

Some *in vitro* experiments suggest that dicumarol may be changed in the body to salicylic acid or some derivative of this acid. Salicylic acid itself gives rise to a hypoprothrombinemia which can be cured by vitamin K.

In the opinion of the Council on Pharmacy and Chemistry† of the American Medical Association, the allowable claims for vitamin K are the following:

Allowable Claims.—Vitamin K, both in its crude form and in certain related naphthoquinones with analogous antihemorrhagic activity, seems to have a specific effect on prothrombin deficiency occurring under certain sets of circumstances:

In primary dietary deficiency of vitamin K which, while admittedly rare, does exist.

In obstructive jaundice, in which vitamin K has proved to have an extraordinary protective effect against hemorrhagic diathesis.

The hemorrhagic state associated with primary hepatic disease is controlled in part, but not entirely, by vitamin K and by the naphthoquinones with analogous activity. The difficulty seems to lie in the fact that the liver cannot utilize the material in the formation of prothrombin, except to a limited degree.

The hemorrhagic states, which exist in connection with certain intestinal diseases such as ulcerative colitis, sprue and celiac disease, characterized by either a loss of continuity of the intestinal tract or by a disturbance of its absorptive surface, are also affected in a specific manner by vitamin K.

In the treatment of the physiological hypoprothrombinemia of the newborn, which exists during the first week of life, the vitamin and its analogues seem to be a specific. It seems now fairly well established that the vitamin itself or the naphthoquinones, when administered parenterally to a woman during labor, in amounts as small as ½ to 2 mg., insures that the newborn infant will have a normal amount of prothrombin in the circulating blood. These doses can also be given parenterally to the newborn infant and will produce the same effect.

A Description of an Experiment to Show How Food Deficiencies (Including Vitamin Deficiencies) Are Determined.—It should be emphasized at the start that it is necessary to specify the subject (animal) under investigation. So long as rats are employed no information regarding the importance of vitamin C can be gathered; for rats do not need vitamin C—perhaps because they can synthesize this substance.

* Pp. 270, 568.

† *New and Nonofficial Remedies*, 1941.

On the other hand, we have already seen how susceptible the guinea-pig (and man) is to vitamin C deficiencies. We know, too, that nicotinic acid, pantothenic acid and inositol are important because investigators turned their attention to the needs of dogs, chicks and mice.

In the experiment under consideration, Woolley, a talented investigator, turned his attention to the nutritive requirements of the guinea-pig. Guinea pigs between one and six days of age (60 to 90 gm.) were fed the appropriate diet and water *ad libitum*. Body weights were taken each week.

The basal ration (ration X)—a highly purified mixture—consisted of vitamin-free casein 18 gm., sucrose 76 gm., salts (comparable to those present in milk) 5 gm., fortified corn oil 1 gm.,* choline 100 mg., inositol 100 mg., thiamine 0.2 mg., riboflavin 0.5 mg., pyridoxine 0.2 mg., nicotinic acid 1 mg., pantothenic acid 1 mg., and ascorbic acid 10 mg.

On this ration guinea-pigs failed to grow normally and died in from two to three weeks.

The results are first tabulated in Table 33.

The substance extracted with 50 per cent alcohol Woolley designates tentatively GPF-1, and that insoluble in the 50 per cent alcohol, GPF-2.

The ration for the assay of GPF-2 (ration Y) consisted of the purified basal ration (ration X) + dried grass (2 per cent of basal ration) + 50 per cent ethanol extract of linseed oil meal. This ration contained all the necessary factors except GPF-2. Various substances were now added to ration Y to determine whether any of them contained the missing factor (experimentally determined by feeding guinea-pigs and noting the average weekly gain). The results of these experiments showed that besides linseed oil meal, 10 per cent of dried grass and 10 per cent of the alcohol-insoluble portion of aqueous beef liver also contained adequate amounts of GPF-2.

TABLE 33.—EVIDENCE FOR TWO NEW FACTORS (Woolley, *J. Biol. Chem.*, **143**, 680).

Supplement.	Amount in ration.	Average weekly gain.
	per cent.	*gm.*
None		7
Dried grass	2	7
Linseed oil meal + dried grass	25	19
50 per cent ethanol extract of linseed oil meal + dried grass	Equivalent to 25	3
50 per cent ethanol residue of linseed oil meal + dried grass	Equivalent to 25	5
Above extract + residue + dried grass	25	24

* To each 100 gm. of corn oil were added 1 gm. (200,000 U. S. P. units) of vitamins A concentrate, 1 gm. of viosterol (10,000 U. S. P. units of vitamin D), 1 gm. of a 40 per cent concentrate of α-tocopherol (vitamin E) from wheat germ oil, and 10 mg. of 2-methyl-1,4-naphthoquinone (equivalent to vitamin K).

The ration for the assay of GPF-1 (ration Z) consisted of the purified basal ration (ration X) + dried grass (2 per cent of basal ration) + the residue left after the linseed oil meal had been extracted with 50 per cent alcohol. Using this ration Z, adding various fractions to it and noting the average weekly gain of the guinea pigs, the following conclusions could be drawn: GPF-1 was not extracted from linseed oil meal by 95 per cent ethanol or by acetone. It was not readily destroyed by alkali. Fuller's earth adsorbed this factor, and it was eluted by barium hydroxide or by dilute alcoholic pyridine.

Evidence for still a third missing factor was obtained when it was found that the addition of both GPF-1 and GPF-2 (in the form of linseed oil meal) gave rise to good growth for the first three to four weeks and that then growth ceased abruptly. Death resulted within a week or two. Dried grass (5 per cent of the ration) did little to help matters.

TABLE 34.—VITAMIN UNITS. (Daniel and Munsell, U. S. Dept. Agric., Publ. No. 275)

VITAMIN A UNITS

International Standard of Reference.—Pure beta carotene conforming to the requirements given for chemical and physical constants. A sample of cod liver oil carefully assayed in terms of the International standard under the supervision of the United States Pharmacopoeia Vitamin Advisory Committee is available as a subsidiary standard.

International Unit.—The activity of 0.6 micrograms (0.6γ) of the International Standard of Reference.

U. S. P. (United States Pharmacopoeia) XI (1936) Unit.—Same as International unit.

VITAMIN B_1 UNITS

DEFINITIONS

International Standard of Reference.—The adsorption product of vitamin B_1 from rice polishings prepared in the Medical Laboratory, Batavia, Java, by the method of Seidell as described by Jansen and Donath.

International Unit.—The International unit is the antineuritic activity of 3 micrograms of crystalline vitamin B_1 hydrochloride. 1 mg. contains 333 U. S. P. or International Units.

VITAMIN C UNITS

DEFINITIONS

International Standard of Reference.—*l*-Ascorbic acid conforming to the specifications given as to chemical and physical constants.

International Unit.—The vitamin C activity of 0.05 mg. of the International standard, *l*-ascorbic acid. This is about one tenth of the daily dose necessary to prevent development of gross macroscopic scorbutic lesions in young guinea-pigs maintained on a scurvy-producing diet.

VITAMIN D UNITS

DEFINITIONS

International Standard of Reference.—A solution of irradiated ergosterol prepared at the National Institute for Medical Research, London. The International Conference recommended that when the supply of this International Standard solution is exhausted, it be replaced by an equivalent solution of pure crystalline vitamin D in olive oil of such strength that 1 mg. contains 0.025 micrograms (0.025γ) of crystalline vitamin D (calciferol). A sample of cod liver oil carefully assayed in terms of the International standard under the supervision of the United States Pharmacopoeia Vitamin Advisory Committee is available as a subsidiary standard.

International Unit.—The vitamin D activity of 1 mg. of the International standard solution of irradiated ergosterol found equal to 0.025 micrograms of crystalline vitamin D.

U. S. P. (United States Pharmacopoeia) XI (1936) Unit.—Same as International unit

For **vitamin values of foods in terms of common measures,** see Hewston and Marsh, Miscellaneous Publication No. 505, U. S. Dept. Agriculture.

These experiments show that guinea-pigs require three (and possibly more than three) dietary essentials besides those which are sufficient for the growth of species such as rats and mice.

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What will undoubtedly prove a standard work is Harris' *Vitamins and Vitamin Deficiencies* (1939), volume **1.**

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Fieser and his group have also done important chemical work in this field. For some of their papers, see *J. Am. Chem. Soc.*, **61**, 2206, 3467 (1939); *Science*, **91**, 31 (1940); *J. Am. Chem. Soc*, **62**, 2861 (1940); *J. Biol. Chem.*, **137**, 659 (1941).

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High cost of vitamin therapy: *J. Am. Med. Assoc*, **126**, 33, 29 (1944). ("Those who are familiar with the true indications for vitamin therapy realize how many hundred million dollars are spent needlessly . . . ")

CHAPTER 9

SYNTHESIS IN THE PLANT KINGDOM

"THE ultimate source of all energy, sustaining life, is the radiant energy of the sun. The energy of the sun's rays is captured by the green dyestuff of plants, the chlorophyll. This radiating energy cannot, as such, support life, for if it were essential, life would fail at night. Thus the energy is used to build up carbohydrate molecules from carbon dioxide and water, the excess of oxygen being sent back into the atmosphere as O_2. In essence these carbohydrate molecules represent small parcels of energy, which can be stored and released by the cell according to needs. The 'unpacking' of these parcels is the reverse reaction, in which the carbohydrate molecule is united again with oxygen to form carbon dioxide and water. This process we call oxidation or combustion." (Szent-Gyorgyi.)

TABLE 35.—SYNTHESIS OF ORGANIC COMPOUNDS IN GREEN PLANTS. (Conant, *Chemistry of Organic Compounds*. By permission of The Macmillan Company, Publishers.)

Raw materials	Primary products	The more important secondary substances.	
		(*a*) Molecular weight less than 1000.	(*b*) Materials of high molecular weight.
CO_2 H_2O Inorganic nitrogen compounds	Sunlight on chloroplast pigments → Sugars, Amino-acids ↓↑ *Reserve materials*; Proteins, fats and oils; Polysaccharides (*e. g.*, starch), hemicelluloses	Polyene pigments Aliphatic acids, alcohols Terpenes Sterols Waxes Phosphatides (*e. g.*, lecithin) Inositol Aromatic hydroxy acids Hydroxy compounds as glucosides (Phenols, complex alcohols) Volatile aldehydes, alcohols, esters, ethers (In essential oils with terpenes) Alkaloids Pyrrole pigments Anthocyan pigments Nucleic acids	Cellulose Hemicelluloses, gums, pectins Resins Rubber Tannins Lignins

Before discussing the digestion and assimilation of foods, some attention should be given to the source of these foodstuffs—to the source of all life, as a matter of fact. The synthesis of organic material in the plant world provides the ultimate source of all nourishment.

Photosynthesis.—The fundamental reaction in this synthetic upbuilding is a photochemical one. Primarily it involves the combination of carbon dioxide and water, in the presence of light and the pigments in the chloroplast (chlorophyll *a*, chlorophyll *b*, carotene, and xanthophyll) to yield carbohydrate and oxygen. Closely associated with this reaction is the production of fats and oils and proteins (here inorganic

nitrogen compounds are necessary). Still other products, the result of various syntheses within the plant, are given in Table 35

The primary photochemical reaction, the reduction of carbon dioxide to sugar, involving the absorption of light by the pigments, is usually represented by a formulation we owe to Baeyer:

$$CO_2 + H_2O \rightarrow \underset{\text{Formaldehyde.}}{H.CHO} + O_2$$

$$6H.CHO \rightarrow \underset{\text{Glucose.}}{C_6H_{12}O_6}$$

Representing these two equations in one stoichiometric reaction, we have:

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$$

The mechanism of this reaction—the parts played by light and by chlorophyll, the intermediate products formed—is still far from clear.

Using heavy oxygen (O^{18}) as a "tracer," it would seem that the oxygen evolved in photosynthesis comes from the water and not from the carbon dioxide.

Even the intermediate production of formaldehyde has been disputed. The rôle of iron in the assimilation process has, as a rule, been neglected in formulating a mechanism; yet without iron, photosynthesis is at a low ebb, due to the small amount of chlorophyll present. This has suggested to Weiss that in the assimilation process the reduction of carbon dioxide involves ferrous ions present in the chloroplast. In the presence of iron, and in the presence of light, the chlorophyll forms a light-absorbing chlorophyll-CO_2 complex, which then takes part in the reduction of carbon dioxide.

In this photosynthetic reaction, two phases have to be considered: a dark-reaction (Blackman reaction) and a photochemical reaction. Conant assumes that chlorophyll is dehydrogenated by carbon dioxide and represents the scheme thus:

1. *Dark-reaction*

$$12 \text{ Chlorophyll} + 6CO_2 + \text{enzyme} \rightarrow 12 \text{ chlorophyll } (-2H) + C_6H_{12}O_6 + 6H_2O$$

2. *Light-reaction*

$$12 \text{ Chlorophyll } (-2H) + \text{light} + 12H_2O \rightarrow 12 \text{ chlorophyll} + 6O_2$$

Solutions of carbon dioxide do not absorb light in the visible region. The photosynthetic reaction involves the absorption and transfer of light by the pigments. Chlorophyll is a photosensitizer.

The imitation of these photosynthetic reactions in the laboratory has been beset with many difficulties. Baly claims to have produced catalysts which when suspended in carbonic acid produce a photosynthesis of organic matter. One such catalyst is kieselguhr coated with aluminum hydroxide in the presence of nickel carbonate containing a small amount of thorium oxide. Using photosensitizers (colored inorganic salts and organic dyes), and working in "tropical

sunlight," Dhar claims to have obtained formaldehyde from carbon dioxide.

If instead of working with artificial catalysts, one uses an extract of plant material or isolated chloroplasts, no photosynthesis of carbohydrates takes place. The photosynthetic reaction in nature requires that "the entire cell structure of the leaf must remain intact" (Nurmia).

Some interesting results have been obtained in the study of photosynthesis by the use of radioactive carbon, obtained by bombarding boron with deuterons:

$$_1D^2 + {}_5B^{10} \rightarrow {}_6C^{11} + {}_0n^1$$

This carbon (C*, radioactive), in the form of C^*O_2 was used. Barley plants were exposed to this C^*O_2 (under conditions of light and darkness), and radioactive carbohydrates and chlorophyll were isolated. Leaves kept in darkness as well as the leaves which were illuminated formed radioactive carbohydrates. The chlorophyll contained radioactivity after exposure to C^*O_2 in the light but not in the dark. Much of the radioactive material found in the plant, however, was water-soluble and not carbohydrate.

A modified view of photosynthesis, based on the use of radioactive carbon, suggests that the first process in photosynthesis is not the formation of a chlorophyll-carbon dioxide complex at all, but the conversion of carbon dioxide to carboxyl in the presence of a large molecule, RH.

$$RH + CO_2 \rightleftarrows RCOOH$$

The photochemical process may now be represented thus:

$$RCOOH + H_2O \xrightarrow{\text{Chlorophyll}} RCH_2OH + O_2$$

The RCOOH formed in the dark is reduced to RCH_2OH in the light. The latter takes on another carbon dioxide molecule, and the cycle is repeated to build up long carbohydrate chains.*

The formation of a hexose from formaldehyde has been accomplished in the laboratory, although to what extent this approaches the synthetic activities of the leaf is problematical. As far back as 1861, Butlerow showed that trioxymethylene, $(H.CHO)_3$, a polymer of formaldehyde, yielded a sweet substance when mixed (and allowed to stand) with hot lime water. This substance reduced Fehling's solution but it could not be fermented by yeast, nor was it optically active. Later a somewhat similar substance—to which the name "formose" was given—was obtained by the direct action of formaldehyde itself with lime water. In 1887, Emil Fischer isolated a sugar, α-acrose, by the action of glycerose and alkali, which proved to be identical with inactive fructose. Glycerose is a mixture of glyceric aldehyde, $CH_2OH.CHOH.CHO$, and dihydroxyacetone, $CH_2OH.CO.CH_2OH$. Fischer made it probable that Butlerow's product and the α-acrose, or

* This view is very debatable. Other hypotheses introduce the formation of α-keto acids and α-hydroxy aldehydes as intermediates.

inactive fructose, were identical. It may be said that, within certain limits, the laboratory has imitated Nature in the production of a hexose from formaldehyde (always assuming that formaldehyde is really first formed in the leaf, which is probable).

Carbon Dioxide as a Building Material in Synthetic Reactions.—Heterotropic, nonphotosynthetic bacteria—organisms which cannot form protein and carbohydrate from inorganic nitrogen and carbon—can "fix" carbon dioxide; that is to say, the carbon dioxide can enter directly into a molecule in the course of synthesis.

That such synthetic reactions involve all forms of life, simple and complex, was made probable when it was suggested that the synthesis of citric acid (p. 325) from oxalacetic acid (p. 326) involved utilization of carbon dioxide. Reactions involving radioactive carbon (p. 206) have confirmed this view.

Chlorophyll.—In the chloroplasts we find four pigments in colloidal combination with complex substances*; two of them, chlorophylls *a* and *b*, are green, and two, carotene and xanthophyll, are yellow. The chemistry of chlorophyll has engaged the attention of chemists for one hundred years. Berzelius, in 1839, attempted to isolate it from leaves, but his treatment was too drastic, and he obtained decomposition products. We owe to Willstätter primarily our knowledge of the substance. He not only isolated chlorophyll in the pure state, but contributed much toward assigning it its present structure.

Chlorophyll consists of two modifications, an *a* and a *b*. In a mixture of petroleum ether and methyl alcohol, component *a* is found in the former and component *b* in the latter. An analysis of these two substances yields the following:

Chlorophyll a, $C_{55}H_{72}N_4O_5Mg$

Chlorophyll b, $C_{55}H_{70}N_4O_6Mg$

Mild hydrolysis produces phytol, $C_{20}H_{39}OH$, an unsaturated alcohol, which constitutes about 30 per cent of the chlorophyll molecule. The *a* component also yields methyl alcohol. The residual molecule is known as chlorophyllin, and chlorophyll *a* itself may be regarded as an ester:

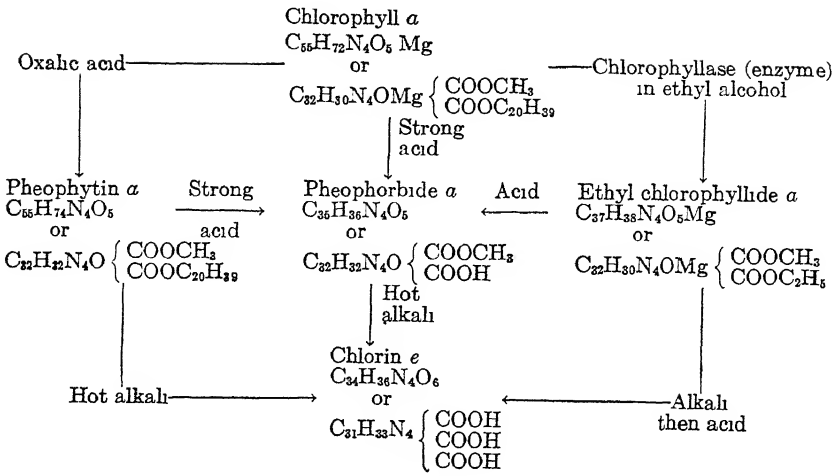
$$C_{32}H_{30}N_4OMg \begin{cases} COOCH_3 \\ COOC_{20}H_{39} \end{cases}$$

The magnesium can be removed from chlorophyll by means of oxalic acid, yielding pheophytin *a*:

$$C_{32}H_{32}N_4O \begin{cases} COOCH_3 \\ COOC_{20}H_{39} \end{cases}$$

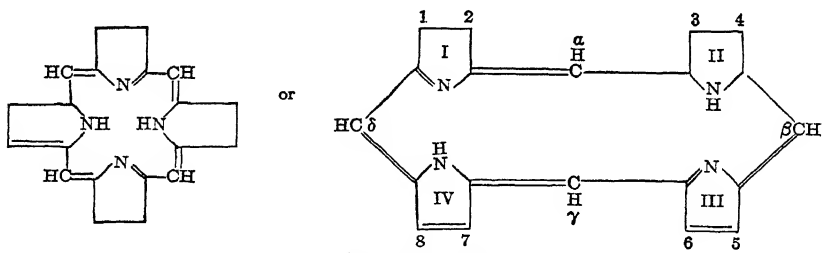
Chlorophyll when heated with alkali first loses its ester groups, and then CO_2. These various phyllin products lose their magnesium by treatment with oxalic acid, giving phytin products. Some of these relationships (and others) are shown as follows:

* The chlorophyll is present in some combination with protein.



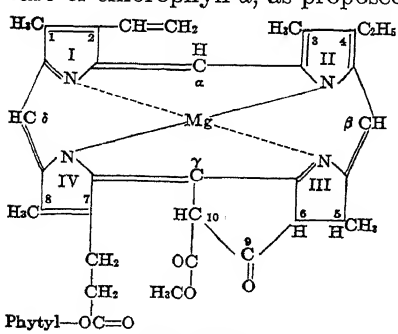
Reduction and oxidation of chlorophyll pigments yield pyrrole compounds (such as hemopyrrole), which suggests the pyrrole nature of chlorophyll itself. Incidentally, a similar series of pyrrole compounds is obtained from hemoglobin.

Drastic alkaline treatment of chlorophyll (and hemoglobin) yields porphyrins, which are red compounds with characteristic absorption spectra, and contain four pyrrole nuclei. Their structure as suggested by Küster is:

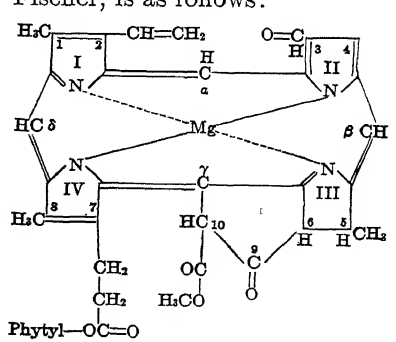


The porphin nucleus.

The unsubstituted nucleus is known as "porphin (see p. 259)." The synthesis of a porphyrin, having the characteristic spectrum of the porphyrins, was first accomplished by Hans Fischer in 1926. The structure of chlorophyll *a*, as proposed by Fischer, is as follows:



Chlorophyll *a*.



Chlorophyll *b*.

The structure of chlorophyll *b* is still somewhat in doubt.

Carotene and Xanthophyll.—The constant association of these two yellow pigments with chlorophyll has suggested that they also play some part in the photosynthetic or in the respiratory process of plants. Carotene, $C_{40}H_{56}$, is a hydrocarbon whose structure has already been given (p. 140). We have seen that it may be looked upon as the mother substance of vitamin A. Xanthophyll, $C_{40}H_{56}O_2$, is a dihydroxy derivative of carotene. It was supposed for a time that carotene and xanthophyll between them have something to do with the transport of oxygen, standing in a relationship comparable to hemoglobin and oxyhemoglobin. But unfortunately for this theory, no simple oxidation converts carotene to xanthophyll, and no simple reduction changes the latter back to the former. Nor is the evidence for their participation in the photosynthetic process any clearer.

The Assimilation of Nitrogen by the Plant.—During the photosynthetic process carbon and hydrogen are absorbed and ultimately utilized by the plant. The nitrogen is derived from various nitrogenous products in the soil.

Using isotopic nitrogen (N^{15}) in the form of ammonium chloride, it can be shown that when this is administered to rapidly growing plants, the isotope is present in various parts of the tissues. The nitrogen of the ammonium is rapidly incorporated into nitrogen of amides, amino acids and proteins.

Using isotopic nitrogen, the ability to "fix" or use molecular nitrogen—attributed from time to time to many biological agents—is in reality limited to a few organisms; among them are *Azobacter vinelandii;* the blue-green alga, *Nostoc muscorum;* and the anaerobic bacterium, *Clostridium pasteurianum.*

References

The problems of photosynthesis are discussed, among others, by Johnson and Myers, *Ann. Rev. Biochem.*, **12,** 473 (1943); Franck, *Sigma Xi Quarterly*, **29,** 81 (1941).

Articles on the chemistry of chlorophyll are by Hans Fischer, *Chem. Rev.*, **20,** 41 (1937); Steele, *Chem. Rev.*, **20,** 1 (1937).

The chloroplast pigments are reviewed by Strain in the *Ann. Rev. Biochem.*, **13,** 591 (1944).

For a general discussion of isotopes in the study of plant growth, consult Brewer, *J. Chem. Educ.*, **18,** 217 (1941). Individual papers are by Ruben, Kamen, Hassid and Perry, *J. Am. Chem. Soc.*, **61,** 661 (1939); **62,** 3443, 3450, 3451 (1940) (radioactive carbon); Wood, Werkman, Hemingway and Nier, *J. Biol. Chem*, **139,** 365 (1941) (heavy carbon); Slade, Wood, Hemingway and Werkman, *Ibid.*, **143,** 133 (1942) (heavy carbon); Ruben, Randall, Kamen and Hyde, *J. Am. Chem. Soc.*, **63,** 877 (1941) (heavy oxygen); Vickery, Pucher, Schoenheimer and Rittenberg, *J. Biol. Chem*, **135,** 531 (1940) (isotopic nitrogen); Norris, Ruben and Allen, *J. Am. Chem. Soc.*, **64,** 3037 (1942) (radioactive hydrogen).

Methods of isolating plant pigments are described by Schertz, *Ind. Eng. Chem.*, **30,** 1073 (1938) (chlorophyll, carotene and xanthophyll); Petering, Wolman and Hibbard, *Ind. Eng. Chem.*, Analytical ed., **12,** 148 (1940) (chlorophyll and carotene); Petering, Morgal and Miller, *Ind. Eng. Chem.*, **32,** 1407 (1940) (carotene); Mackinney, *J. Biol. Chem.*, **132,** 91 (1940) (chlorophyll).

Chromatographic adsorption analysis is particularly adapted to this field. A very good account of this method of analysis is given by Strain, *Ind. Eng. Chem.*, **14,** 245 (1942). A standard book on chromatography is by Zechmeister and Cholnoky (1941).

References to chlorophyll as part of a pigment-protein complex in the green leaf may be found in papers by Smith, *Science*, **91,** 199 (1940), *J Gen Physiol*, **24,** 565 (1941), Anson, *Science*, **93,** 186 (1941), Fishman and Moyer, *J. Gen. Physiol*, **25,** 755 (1942); French, *Ibid*, **23,** 469, 483 (1940).

Ruben, *J Am. Chem. Soc*, **65,** 279 (1943), connects the photosynthetic process with phosphorylation

Photochemical reactions in algae are discussed by Gaffron, *J Gen Physiol*, **26,** 195 (1942), Gaffron and Rubin, *Ibid*, **26,** 219 (1942), and Gaffron, *Ibid.*, **26,** 241 (1942).

Chlorophyll *d*, a green pigment of red algae, is described by Manning and Strain, *J. Biol Chem*, **151,** 1 (1943).

For the detection of nitrogen fixation with isotopic nitrogen, see Burris, Eppling, Wahlin and Wilson, *J. Biol. Chem*, **148,** 349 (1943).

CHAPTER 10

DIGESTION

In general, foods need to be hydrolyzed, to be simplified, chemically, before they can be assimilated by the body. This is true of the carbohydrates other than the monosaccharides, and of the fats and proteins. It is not true of water and the various inorganic salts; for they pass through the digestive tract without undergoing any changes,

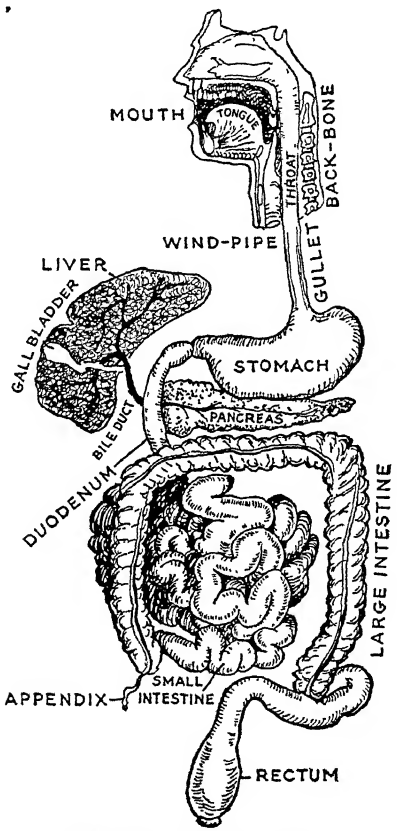


Fig. 56.—Digestive tract.

and they are absorbed in their original form. The simplification of the carbohydrates, fats, and proteins is accomplished by a series of hydrolytic changes brought about by enzymes. These changes are carried on in the digestive tube, which includes the mouth, the esophagus, the stomach, and the small and large intestines (Figs. 56, 57). Secretions from the pancreas and the bile find their way into the small

intestine, and, as we shall see, play important rôles in the digestive process.

As so much of digestion deals with the activity of enzymes, the reader will do well to reread Chapter 6, which deals with the general properties of enzymes.

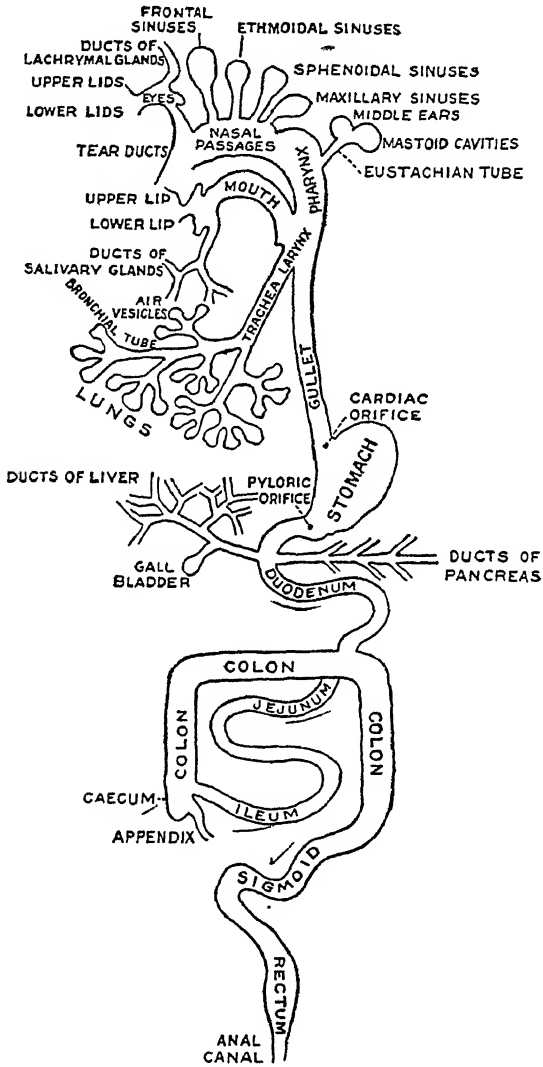


Fig. 57.—Digestive tract [Bundy's *Anatomy and Physiology* (1940), revised by Weeder, p. 15, after Gerrish, modified.] The Blakiston Co.

SALIVARY DIGESTION

Salivary digestion deals largely with the action of ptyalin, an amylase, on starch. The preliminary mastication, involving the breaking up of food particles by the teeth, is a desirable step. The ptyalin is found in saliva. Saliva represents a mixed secretion from the relatively

large salivary glands, and also from accessory glands. The salivary glands consist of the parotid, the submaxillary, and the sublingual, and through their ducts they pour their secretions into the mouth. The flow of saliva is regulated by a reflex stimulation of the secretory nerves. Approximately from 1 to 1½ liters of saliva may be secreted by an individual in the course of twenty-four hours.

Composition.—This saliva contains about 99.5 per cent of water. The solid material includes the ptyalin, several proteins (of which mucin is the most important), a number of substances found in blood and urine (such as ammonia, urea, uric acid, cholesterol, calcium, sodium, potassium, magnesium, phosphate, chloride, bicarbonate), and strangely enough, thiocyanate, the origin of which is shrouded in mystery. The average *p*H of unstimulated saliva may vary fairly considerably, although as a rule it is around 6.8

The fact that saliva contains many of the constituents found in blood has suggested the possibility that the determination of such constituents in saliva may have diagnostic value; but the irregular fluctuation, and a failure to find any constant relationship between the constituents of blood and saliva, have not led to any practical results.

Action of Ptyalin.—The ptyalin (also known as salivary amylase) acts on starch* producing a series of ill-defined products: soluble starch, erythrodextrin, achrodextrins, and maltose. The starch and soluble starch give a blue color with iodine, the erythrodextrin gives a red color, and the achrodextrins and maltose give no color. Maltose is the only product which reduces Benedict's solution, unless some glucose is formed (see below) which would, of course, also reduce Benedict's solution.

Actually, the process of hydrolysis is more complex. Some maltose appears even at the erythrodextrin stage. This has been attributed by some to the complex nature of the starch itself (see p. 23).

The optimum for amylase activity is a *p*H of 6.6. The activity of the enzyme is stimulated by the presence of halogens, particularly the chloride ion. The removal of this ion by dialysis renders the ptyalin inactive. At a *p*H of 4 or below the enzyme is rapidly destroyed. However, when the food reaches the fundus part of the stomach, salivary digestion may still proceed for fifteen to thirty minutes, due to the slow accumulation of acid and to the partial neutralization of the acid by a temporary combination with the protein of the food.

The claim has been made that a small amount of glucose is also formed during the course of the hydrolysis of starch, due to the presence of an enzyme, maltase, which converts maltose into glucose. Stark is of the opinion that the products of the action of ptyalin on starch include glucose, maltose and "an array of nonfermentable copper-reducing polysaccharides."

The pancreatic amylase (p. 225), which finds its way into the small intestine, continues the hydrolysis of any undigested starch.

In the tissues we find an enzyme, phosphorylase, which can also

* The ptyalin can also act on glycogen.

break down starch and glycogen (p. 91). This action is not to be confused with the action of amylase on starch and glycogen—whether the amylase be ptyalin, or pancreatic amylase or amylases which have their origin in the vegetable kingdom.

Neither ptyalin nor any of the other amylases have been isolated as yet; but there is reason to believe that the amylases are also proteins.*

Estimation of Ptyalin Activity.—The rate of starch hydrolysis (and the activity of the ptyalin) may be estimated by determining the achromic point—the point at which iodine fails to give a color with the product—or by determining the extent of reduction. A more carefully controlled estimation would include the achromic point, the residual polysaccharide, the total reducing power, and the reducing power after the precipitation of the dextrins by alcohol. Using such methods, Glock found that the relative rates of hydrolysis were different with different starches.

Mucin.—Mucin, the protein present in largest quantity, gives to saliva its "ropy" consistency. It is present as a soluble alkaline (probably potassium) salt and can be precipitated by the addition of acid. It has been classified as a glycoprotein, because on hydrolysis it yields glucosamine as one of its products. However, in addition to glucosamine, we also get glucuronic, acetic, and sulfuric acids.†

Tooth Decay (Dental Caries).—It has been suggested that in addition to its function as a digestive agent, the saliva is also important in its influence on the possible development of dental caries. This wellnigh ever-present disease—85–95 per cent of people in "civilized" countries suffer from it—is of two kinds: in the one, common to young people, caries occurs in the pits and fissures of the crown or near points of contact of adjacent teeth; in the other, prevalent among older people, the smooth surfaces of the crown or exposed roots are attacked. The first variety is the more common, occurring very often during the period of eruption of the teeth.

The various centers of attack, the pits and fissures of the crown and the contact points, are precisely centers where food particles are likely to be deposited. The action of bacteria on such food particles may cause the production of acid. It is believed—and this is advanced as a theory of the origin of dental caries—that first the enamel on the surface of the tooth and then the dentin underneath are dissolved by such acids.

If such a theory is sound, then, one might expect a difference be-

* As has been pointed out (p. 94), amylases are found in the animal and vegetable kingdoms. A high concentration of amylase is found in the pancreas of higher animals. In some species (man, pig, rat), a fairly high concentration of the enzyme is found in the saliva. In herbivora, in general, the concentration of amylase in the saliva is low.

Amylases are also found in blood plasma, leucocytes, etc.

† The mucins have been classified into a number of groups, depending upon their chemical make up: mucins proper, sulfomucins, chondroproteins and mucoproteins. The mucin of saliva comes more under the heading of the mucoproteins—glycoproteins whose prosthetic grouping is of the nature of mucoitin sulfuric acid (glucosamine, glycuronic, acetic and sulfuric acids).

tween the neutralizing power of saliva derived from a patient suffering with caries and the saliva derived from a caries-free individual, assuming that saliva has some access to the regions where decay occurs. Furthermore, since the enamel is rich in the elements calcium and phosphorus, an analysis of such elements in saliva may prove revealing.

Karshan and Krasnow did find that saliva, stimulated by chewing paraffin and obtained from individuals showing no caries, had a neutralizing power (as revealed by titration with acid), which, in group averages, was 10 per cent greater than that obtained from persons suffering with caries. A much greater difference in mean values between the two groups was found by Hubbell. However, when unstimulated saliva was used, no such difference could be noted.

This neutralizing power of saliva can be measured in another way: the carbon dioxide capacity of the fluid can be determined. As this topic is discussed under blood (Chap. 13), it will be enough to say at this point that the amount of carbon dioxide held in combination with alkali as sodium or potassium bicarbonate varies directly with the amount of such alkali present. If, now, acid be added, and the amount of carbon dioxide which is evolved be determined, the greater the volume of such gas evolved, the larger the amount of alkali in the fluid. Using many subjects, and taking average values, the results with stimulated saliva were: caries-free, 31 (cc. of CO_2 per 100 cc. of saliva); arrested caries, 30.2; active caries, 19.5.

Turning next to studies dealing with the content of calcium and phosphorus in saliva, a number of workers were able to show that the mean values for total calcium and inorganic phosphate were higher in caries-free than in active-caries groups.

Apparently, there is some correlation between the penetration of enamel and the development of caries, on the one hand, and the composition of saliva, on the other. However, these studies tell us little, or nothing at all, as to possible methods of preventing such tooth decay. Attempts have been made, with indifferent success so far, to change the diet in the hope that it might influence the composition of the saliva.* (For the effect of *fluorine* on tooth decay, see p. 431.)

Salivary Calculus.—This abnormal concretion—formed on the teeth and sometimes in a salivary duct—contains calcium phosphate as its principal inorganic constituent. The idea has arisen, very naturally, that the concentration of calcium and phosphate in saliva may be related to such deposits. Furthermore, the precipitation of calcium phosphate would be a function of the *p*H of the medium. Actual experiments indicated that the mean value for the amount of calcium in the calculus-free group was lower than that in the calculus group. To a certain extent, this was also true of the content of phosphate, which was lower in the calculus-free group. The studies in *p*H showed no such clear-cut differences.

* Turner and Crane claim that, under certain conditions, starch hydrolysis by saliva takes 45 minutes for completion, provided dental caries are absent. The saliva of patients with dental caries hydrolyzes starch much more quickly: with four to six cavities the hydrolysis is complete in 19 minutes; with ten to twelve cavities, in 7 minutes; with 20–30 cavities, in less than 2 minutes.

GASTRIC DIGESTION

Digestion in the stomach involves, primarily, the action of the enzyme pepsin and hydrochloric acid on protein, yielding hydrolytic products such as proteoses and peptone. The enzyme rennin is also present; its function is to curdle milk. Some lipase, a fat-splitting enzyme, may also be found. The food, mixed with saliva, and formed into a bolus, passes through the pharynx and esophagus into the stomach (Fig. 58). There it gradually comes in contact with the pepsin

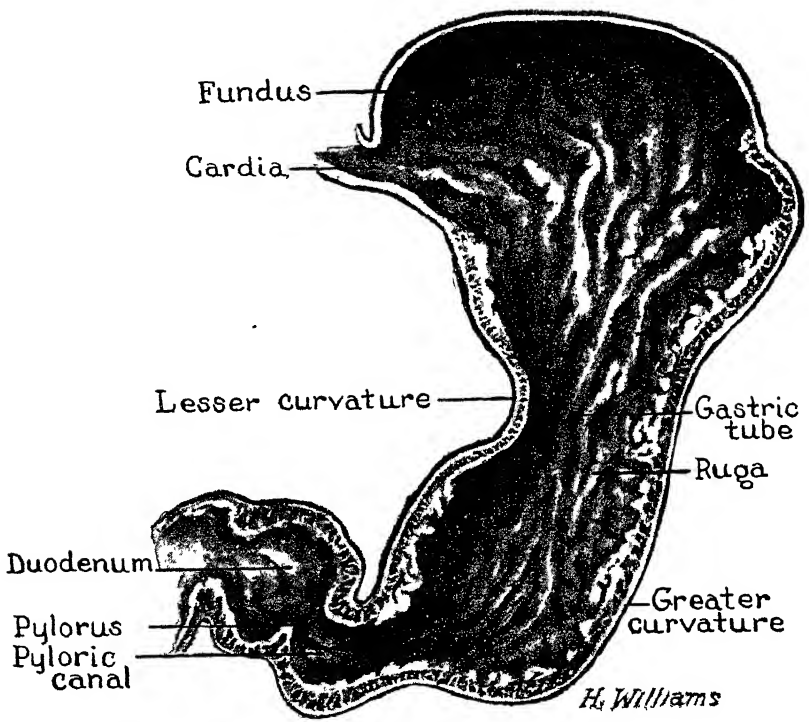


Fig 58.—Vertical section of stomach. (Francis, *Fundamentals of Anatomy*, C. V. Mosby Co.)

and the acid. For some time, however, considerable starch digestion continues in the fundus part of the stomach.

As early as 1783, Spallanzani detected the acidity of gastric juice and also noted that it had the power of dissolving meat. He introduced food in perforated metal capsules into the stomach and recovered their contents by strings attached to the capsules. In 1833 Beaumont, an American physician, published his "Experiments and Observations on the Gastric Juice and the Physiology of Digestion," in which he described various experiments performed on a patient who, as a result of a gunshot wound, found himself with an opening from the stomach to the exterior. This publication laid the foundation for much of our knowledge of gastric digestion. He described the digestibility of different foods in the stomach, confirmed the presence of hydrochloric

acid (first noted by Prout), compared *in vivo* with *in vitro* experiments, and made an exhaustive study of the motions of the stomach.

Further contributions on the composition of normal gastric juice were made by Heidenhain, who cut away the fundic or pyloric end of the stomach and created an opening to the exterior. The secretion of this isolated sac was then studied. The experimental technic was subsequently vastly improved by Pavlov.

Gastric Juice.—In the walls of the stomach one finds two types of glands, those at the pyloric end composed of one type of cells known as the chief cells, and those in the central part of the stomach and elsewhere, consisting of chief cells and parietal (border or oxyntic) cells. These glands secrete what ultimately appears as gastric juice. It is believed by some that the hydrochloric acid is produced by the oxyntic cells, while the chief cells throughout the stomach produce the other constituents.

The flow of gastric juice is controlled by nerve fibers. In one of his classical experiments dealing with "sham feeding," Pavlov divided the esophagus and brought the ends to the skin. The animal ate and discharged its food through this opening, without any of the food finding its way into the stomach. Nevertheless, an abundant flow of gastric juice was induced so long as the vagi were intact; but the flow was interrupted when the nerves were cut. Pavlov named this type of secretion a "physical" one.

However, there is apparently a "chemical" as well as a "psychical" influence. It is possible to extract from the gastric mucosa a substance to which the name "gastrin" has been given, but which some regard as identical with histamine (p. 232), which when injected into the blood causes a flow of gastric juice. The "gastrin" (or the histamine?) plays the rôle of a hormone.

Composition of Gastric Juice.—The stomach secretes some 2 to 3 liters of gastric juice in twenty-four hours. This juice, like saliva, usually contains water to the extent of more than 99 per cent. The material consists of the enzymes pepsin, rennin and lipase, hydrochloric acid (around 0.5 per cent), the chlorides of sodium and potassium, phosphates, etc.

Origin of Hydrochloric Acid.—It is a remarkable fact that a mineral acid of the type of hydrochloric, with a concentration up to 0.5 per cent, should be made in the stomach from an approximately neutral fluid. No other secretion manufactured by the body approaches the gastric juice in high acidity. What is the origin of this hydrochloric acid? It is not hard to assume that the chloride part of the acid has its origin in the chloride of the blood; but no satisfactory explanation has yet been offered for the origin of the comparatively high hydrogenion concentration. The theories advanced are legion. We will refer to but a few.

It is now generally believed that the acid is formed in the fundus part of the stomach, more particularly in the parietal cells which are found there. The hydrochloric acid, or some organic equivalent, is formed inside the cell. If we assume an organic chloride, then we must

further assume a hydrolysis and a reabsorption of the nonacid portion. But if we assume no such organic combination, then the production of hydrochloric acid creates other difficulties. Several authors have advanced the hypothesis that the action of an acid phosphate on a chloride might liberate the acid, which, once formed within the cell, would quickly diffuse through the cellular membrane. Others have supported an idea developed by Mathews that the dissociation of ammonium chloride is the primary cause of acid production.

In forming the acid secretion, the cells of the gastric mucosa (of dogs) raise the hydrogen ion concentration from *p*H 7.4 (the hydrogen ion concentration of the blood) to *p*H 1–2, the hydrogen ion concentration of the acid secretion.

The chloride ion concentration is increased from 0.11 M in plasma to 0.17 M in the secretion.

A mechanism for the secretion of acid has been suggested by Davenport (Fig. 59). In the parietal cells carbonic acid is formed, which dissociates and is catalyzed in this reaction by the enzyme, carbonic anhydrase (p. 306). Chloride ions pass from the plasma through the cells and into the secretion. These chloride ions which are removed from the plasma are replaced by the bicarbonate ions formed in the cells at the time when hydrogen ions are formed.

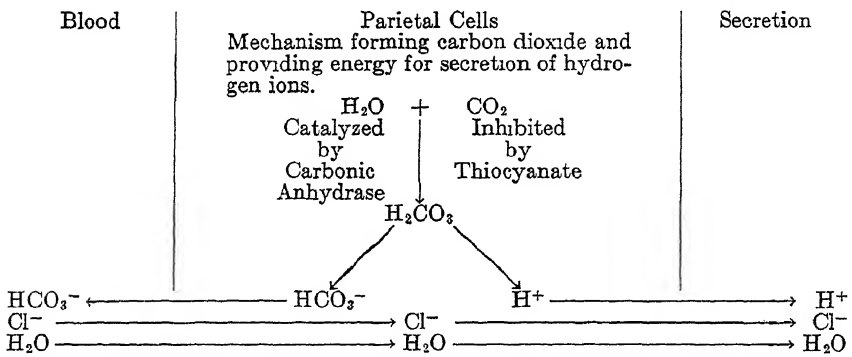


Fig. 59.—Formation of Hydrochloric Acid in the Stomach (Davenport and Fisher, *Am. J. Physiol.*, **131**, 165.)

Pepsin and Pepsinogen.—The active proteolytic enzyme in the gastric juice is pepsin, which, however, is quite inactive except in acid solution. (For several details regarding pepsin, see pages 96, 97.) Langley pointed out many years ago that in the gastric mucosa the enzyme existed in an inactive form which was more resistant to alkali than the pepsin. The inactive form of the enzyme was given the name *pepsinogen*, and the general name *zymogen* was given to an inactive form of an enzyme.

In the meantime, both pepsin and pepsinogen have been isolated in crystalline form, and both show the general characteristics of proteins. Pepsinogen has no proteolytic activity, but is converted into active pepsin in slightly acid solution.

Pepsinogen in crystalline form has been isolated by Herriott from

swine stomach mucosa by extraction with bicarbonate-ammonium sulfate solution,* followed by the precipitation of the soluble pepsinogen at a higher concentration of ammonium sulfate. The precipitate was dissolved in water and treated with copper hydroxide at *p*H 6.0, which adsorbed the pepsinogen; the latter was eluted with phosphate buffer of *p*H 6.8. This treatment with copper hydroxide was repeated. To crystallize the pepsinogen, it was first precipitated with 0.7 saturated ammonium sulfate, then dissolved in nine volumes of 0.4 saturated ammonium sulfate at *p*H 6.3 (M/10 phosphate buffer), and kept for twenty-four hours at 10° C. (Table 36).

TABLE 36.—COMPARISON OF PROPERTIES OF SWINE PEPSINOGEN AND PEPSIN.

Crystalline form.	*Pepsin* Hexagonal bipyramids.	*Pepsinogen.* Needles.
Catalytic activity. .	+	−
Isoelectric point ..	*p*H 2 7	*p*H 3 8
Molecular weight. . .	38,000	42,000
*p*H stability ..	*p*H 2–6	*p*H 6–9
Analysis (per cent):		
Carbon . ..	51.7	52 8
Hydrogen . . .	6.8	6 9
Nitrogen	14.0	13.9
Sulfur	0.45	0.4
Phosphorus	0 09	0.08
Chlorine .	0.00	0.00

Pepsinogen is converted into pepsin at *p*H below 6.0. At *p*H 4.6 the reaction is autocatalytic—pepsin, that is, catalyzes the reaction and so produces more of itself. Crystalline pepsin may be obtained in this way or from commercial pepsin, as described in Chap. 6.

The conversion of pepsinogen to pepsin at *p*H 1.0 to 5.0 is accompanied by the production of a number of polypeptides. One of these polypeptides has an inhibiting action on pepsin at *p*H 5.0–6.0. Herriott has isolated this "inhibitor" and finds it to have a molecular weight of about 5000, with an isoelectric point at *p*H 3.7. It contains arginine but no tryptophan and is destroyed by pepsin at *p*H 3.5.

Pepsin, a Protein.—That pepsin, like the other enzymes which ha been isolated, is a protein is now well established. There is, however, still some question as to whether the enzyme preparations are pure proteins. Koch has prepared amorphous pepsin with a higher activity than crystalline pepsin. Northrop found some samples of crystalline pepsin contained about 50 per cent of inert protein and all of them contained some other enzyme particularly active on gelatin. Herriott showed that pepsin prepared from different sources differed in activity. He proved that this difference was primarily due to the presence of at least two active proteins, with different solubilities.

The more soluble and more active component Herriott and

* For example, 3400 gm. of minced, frozen mucosae was mixed with four times its weight of 0.45 saturated ammonium sulfate in M/10 sodium bicarbonate.

Northrop isolated by repeated extraction with 0.6 saturated magnesium sulfate at *p*H 5.0

The more insoluble (and less active) component was prepared by repeated precipitation with 0.45 saturated magnesium sulfate at *p*H 5.0. The homogeneity of this substance is still in doubt.

For the determination of peptic activity several methods are available: the use of the Van Slyke amino titration (p. 58) and the Sörensen formol titration (p. 59). Northrop has introduced two others. One is based on the increase in conductivity as hydrolysis of the protein proceeds; and the other is based on the rate of change in viscosity of gelatin during digestion A colorimetric method, using hemoglobin as a substrate, has been perfected by Anson and Mirsky. In this method—which, like many others, can be used for determining not only peptic activity but also the activity of other proteolytic enzymes, like trypsin, papain, cathepsin—denatured hemoglobin is digested by pepsin under standard conditions; the undigested hemoglobin is precipitated with trichloroacetic acid; and the amount of unprecipitated protein split products (a measure of the amount of pepsin present) is estimated colorimetrically with Folin's phenol reagent—a phosphotungstic-phosphomolybdic acid—which gives a blue color with the tyrosine and tryptophan present in the hydrolyzed extract.

The great advantage of Anson and Mirsky's method is that "hemoglobin, unlike casein and gelatin, is a reproducible substrate. Different batches of hemoglobin are digested at the same rate by a given proteinase solution."

The optimum *p*H for pepsin action is around 2. What is important, as Northrop has shown, is the hydrogen ion concentration, and not any particular acid. At equal *p*H's, the rate of peptic digestion of various proteins is the same in solutions of hydrochloric, nitric, sulfuric, oxalic, citric, and phosphoric acids.

The Products of Peptic Hydrolysis.—It is probable that during the few hours that the food stays in the stomach, peptic hydrolysis of proteins produces the rather ill-defined proteoses and peptone, but that no amino acids are produced. By incubating protein with an artificial pepsin-hydrochloric acid mixture for some twenty-four hours, it is possible to show the production of some amino acids; but this can hardly apply to gastric digestion *in vivo*.

The first product of peptic hydrolysis is said to be "acid-metaprotein," a soluble protein which precipitates on the careful addition of alkali, and which coagulates when the precipitate is heated. Further hydrolysis produces proteoses and peptone. Proteoses are precipitated with ammonium sulfate: one-half saturated ammonium sulfate precipitating the primary proteoses, and the fully saturated solution precipitating the secondary proteoses. In the filtrate we find peptone, which can be precipitated by certain alkaloidal reagents like tannic acid. These ill-defined stages of peptic hydrolysis differ somewhat in their reaction to the biuret reagent: the primary proteins give definite violet colors, whereas peptone mixed with the biuret reagent is rose-red in color.

Rennin (also known as *rennet* or *chymosin*).—Another enzyme elaborated by the glands of the gastric mucosa* is one which coagulates milk. This enzyme, known as rennin, acts on the casein of the milk. It is believed that the rennin acts on the casein to change it to some soluble product, to which the name "paracasein" has been given. In the presence of calcium, the paracasein becomes the milk clot.

Commercial peptic preparations—and preparations of various proteolytic enzymes—show not only proteolytic properties, but also the property of clotting milk. This had led to a view that pepsin and rennin were one and the same enzyme, and that within the pepsin molecule certain groupings exhibited rennin properties. Tauber and Kleiner were able to separate the rennin from pepsin by a combination of isoelectric and fractional precipitations. This rennin has an activity of 1:4,550,000 when skim milk and calcium chloride are used as substrates, but shows no peptic activity at *p*H 2 (using the formol method). Crystalline pepsin, however, has a rennet activity of 1:800,000.

By various fractional precipitations and crystallizations, Berridge has obtained a crystalline product capable of clotting ten million times its weight of milk in ten minutes.†

Sumner points out that a method of distinguishing rennin from pepsin is to make use of the fact that rennin is not appreciably destroyed if kept at *p*H 9 for a short time, whereas at this *p*H pepsin is rapidly inactivated.

That rennin, like pepsin, is first formed in the inactive, zymogen, condition (prorennin) has been suggested by Kleiner and Tauber, who, using aqueous calcium carbonate, *p*H 7.4, were able to extract from the mucosa of the fourth stomach of the calf (a rich source of rennin) material which is quite inactive when left for two to three days at 37° C., but becomes active at *p*H 5 or below.

Lipase.—This is an enzyme which hydrolyzes fats. Its action at *p*H 1 to 2, the normal reaction of gastric juice, is very slight, but Willstätter has shown that its optimum *p*H is about 5, at which acidity hydrolysis becomes more apparent. In any case, it would seem that under normal conditions, gastric lipase is of little physiological importance. As we shall see presently, the important fat-splitting enzyme is found in the pancreas.

Gastric Analysis.—An analysis of gastric contents is of importance in clinical diagnosis.

The quantity of material found normally in the fasting stomach (interdigestive period) is about 50 cc. An increase above this amount may be due to retention or regurgitation from the duodenum.

Freshly secreted gastric juice is usually colorless. If yellow or green, it may indicate the presence of bile, due to intestinal obstruction. If red or brown, it may mean the presence of blood, which can be confirmed by the benzidine test (p. 272).

* Probably present in relatively large quantities only in the stomach of young animals.

† Commercially rennin plays its role in the making of cheese and in the preparation of junket.

Such blood may suggest lesions such as carcinoma of the stomach, peptic ulcer, etc.

The presence of organic acids, such as lactic acid, suggests bacterial action. In the presence of much free acidity, bacterial activity would be prevented. This means, too, that little acid is present (hypochlorhydria); and this, in conjunction with the presence of lactic acid, may suggest carcinoma of the stomach.

Achlorhydria, the absence of free acid, together with the absence of pepsin, may suggest pernicious anemia, gastric carcinoma (cancer of the stomach), etc.*

To determine gastric acidity, we measure the number of cc. of 0.1*N* NaOH required to neutralize 100 cc. of gastric contents. The *free* HCl may show an average value of 18 5; which means that 18.5 cc. of 0.1*N* NaOH are needed to neutralize 100 cc of gastric contents. The free HCl may also be expressed as grams of HCl per 100 cc. of gastric contents; an average value would be 0.0675 gm.

By *total acidity* is meant free HCl, HCl combined with protein, acid salts (phosphates and carbonates) and organic acids (lactic, butyric, etc.) Its value averages 30; that is, 30 cc. 0.1*N* NaOH to neutralize 100 cc. of gastric contents; or an average of 0.1095 gm. of HCl

The methods for free and total acidity are identical, except that different indicators are used For instance, Töpfer's reagent (dimethylaminoazobenzene), an indicator with a *p*H range of 2 9 to 4.0, is frequently used for the determination of free HCl; and phenolphthalein, with a *p*H range of about 8 to 9, is used for determining total acidity.

Stomach contents are withdrawn after stimulation by the introduction of foods (test meals), alcohol, or the injection of histamine.

Hypoacidity (hypochlorhydria) may suggest carcinoma of the stomach, chronic constipation, chronic gastritis (inflammation of the stomach), chronic appendicitis, etc.

Hyperacidity may suggest gastric ulcer (peptic ulcer), duodenal ulcer, cholecystitis (inflammation of the gall-bladder), etc.

Pernicious Anemia.—The successful use of liver in the treatment of pernicious anemia (see p. 260) was followed by an explanation by Castle of the mechanism of the reaction. The maturing factor necessary for the production of erythrocytes is formed, according to Castle, by the interaction—in the alimentary canal—of an *extrinsic* factor from food ingested with an *intrinsic* factor developed by the *normal* stomach. The maturing factor (M.F.) is stored chiefly in the liver.

The intrinsic factor, indispensable for the development of M.F., is found in *normal* gastric juice. The extrinsic factor, found in foods, was thought for a time to be one or more of the *B* vitamins; but this idea has now been given up.

Pernicious anemia may, therefore develop with a gastric juice that is *abnormal*, as when HCl and pepsin are absent (achylia).

* *Achylia* connotes the absence of both HCl and the gastric enzymes.

PANCREATIC DIGESTION

After a while, the food in the stomach, now in more or less liquid form (chyme), passes into the small intestine. Here it is attacked by intestinal juice (*succus entericus*), pancreatic juice, and bile. The latter two find their way into the duodenum via the pancreatic and bile ducts, respectively, which open by a common orifice into the small intestine (Fig. 60). For purposes of convenience, we shall discuss these three secretions separately.

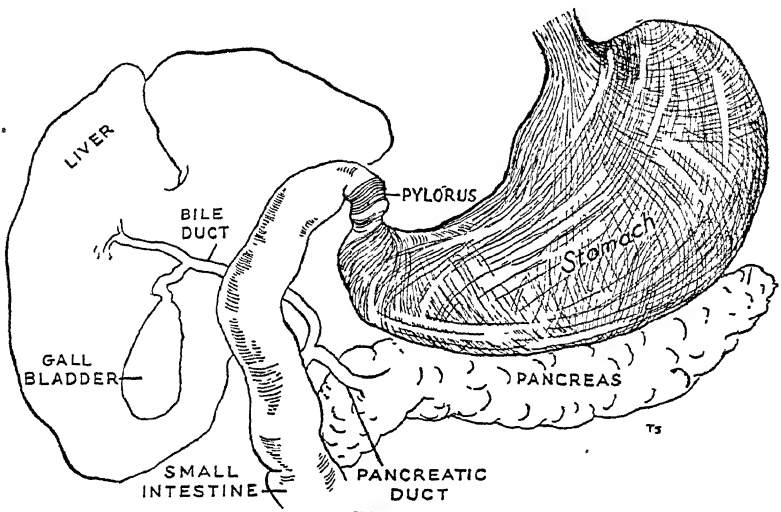


Fig. 60.

Pancreatic Juice.—This is, in a sense, the most important of the digestive juices. It contains enzymes which split proteins, an enzyme which hydrolyzes starch, and another which hydrolyzes fats. As in the case of pepsinogen, the proteolytic enzymes as they first appear are in the inactive, or zymogen, condition. One of them, trypsinogen, is activated by a substance usually present in the intestinal juice and referred to as *enterokinase*. Another proteolytic enzyme, chymotrypsinogen, seems to be activated by trypsin. (Refer, again, to Chap. 6 on Enzymes.) Carboxypeptidase, one of the constituents of "erepsin" (p. 224) is also found in pancreatic juice.

There is evidence to point to the fact that the pancreatic juice also contains some factor which plays a part in the metabolism of lipids (p. 337). The blood lipids (cholesterol, free and esterified, phospholipids and total fatty acids) drop markedly when depancreatized dogs are given insulin (p. 315) and a "normal" diet. The same result is obtained by completely occluding the pancreatic ducts. The condition can be markedly improved by the administration of pancreatic juice or the addition of pancreas to the diet (see p. 337).

Enterokinase.—Trypsinogen can be autocatalytically transformed into active trypsin at *p*H 7.0 to 9.0 (refer to Chap. 6). However, this is not the only way in which the inactive zymogen can be transformed into the active enzyme. Pavlov discovered a substance in intestinal

juice (which he called *enterokinase*) which could transform trypsinogen into trypsin. Pavlov was of the opinion that enterokinase itself was an enzyme. A careful study by Kunitz has confirmed this point of view. He finds that crystalline trypsinogen is transformed into trypsin by enterokinase in the range of *p*H 5.2 to 6.0.

The process of conversion by enterokinase, Kunitz finds, follows the course of a catalytic unimolecular reaction (see Chap. 6), "the rate of formation of trypsin being proportional to the concentration of enterokinase added, and the ultimate amount of trypsin formed being independent of the concentration of enterokinase."

By fractional precipitation with ammonium sulfate, using the proper *p*H conditions, Kunitz has prepared some highly concentrated solutions of enterokinase.

Secretin.*—In 1902 Bayliss and Starling showed that the stimulation of pancreatic juice was due to a substance in the lining of the intestinal wall to which they gave the name of *secretin*. An extract containing the substance could be obtained from the intestinal mucosa which, when injected, caused a copious flow of pancreatic juice. This chemical messenger, acting via the blood, was given the general name of *hormone* ("to excite") (see Chap. 24).

The discoverers claimed that the hormone was present in an inactive (prosecretin) condition, and that the acid coming from the stomach converted the inactive into the active (secretin) form. Once produced, this secretin finds its way into the blood and then stimulates the pancreas.

Hammarsten is of the opinion that secretin is a fairly complex polypeptide.

Ivy believes that blood serum contains an enzyme, secretinase, the action of which is to remove secretin from circulation and thereby regulate pancreatic secretion. At the time when secretin stimulates pancreatic secretion, the gallbladder empties (of its bile). This is due to a contraction of the musculature of the gallbladder by a hormone, cholecystokinin (p. 226).

Ivy, in confirmation of Raper's work, states that there are *two* hormonal factors controlling the external secretion of the pancreas: one is secretin, which stimulates the production of pancreatic fluid; and the other is another hormone, to which the name *pancreozymin* has been given, which stimulates enzyme production by the pancreas.

The Proteolytic Enzymes.—Until quite recently, it was the custom to refer to two enzymes, trypsin and erepsin. Trypsin, it was said, attacked native proteins and hydrolyzed them down to the amino acid stage. Erepsin, it was said, was an enzyme which acted primarily on proteoses (and peptones?), hydrolyzing them to amino acids. Erepsin was regarded as completing the work of pepsin.

The situation has changed considerably although the atmosphere is still confused. The "erepsin" which is found both in the pancreas and in the small intestine is certainly a mixture of enzymes, one of them, the carboxypeptidase, being normally present in pancreatic juice.

* See also p. 523.

Trypsin when pure does not hydrolyze proteins beyond the proteose or polypeptide stage. The further hydrolysis is undertaken by a mixture of enzymes—carboxypeptidase (1) in the pancreatic juice, and aminopeptidase (2) and dipeptidase (3) mainly in intestinal juice. (1) acts on polypeptides provided their carboxyl group is free. (2) acts on polypeptides provided their amino groups are free. (3) acts on dipeptides hydrolyzing them to amino acids (see p. 91).

Besides trypsin, there is, according to Northrop, still another proteolytic enzyme in pancreatic juice which he calls chymotrypsin (see Chap. 6). Both trypsin and chymotrypsin act on proteins to produce proteoses and peptones, but they are distinguished in two ways: first, chymotrypsin has a much greater milk-coagulating power than trypsin second, whereas trypsinogen is activated by enterokinase to form trypsin, the chymotrypsinogen is activated by trypsin into chymotrypsin (see also p. 98).*

Pancreatic Amylase.—This enzyme is, in general, quite similar to the ptyalin of saliva (p. 213). By adsorption on alumina gel at *p*H 6.0 and elution at *p*H 7.3, Sherman has obtained extremely active fractions which still show the typical properties of a protein.†

Lipase.—This important enzyme hydrolyzes fats into fatty acid and glycerol. Enzyme activity may be estimated by titrating the free fatty acid produced with standard alkali. Using protein-precipitating agents, King has succeeded in purifying a sample sufficiently to regard it as protein.

Pathology of the Pancreas.—The two lesions commonly observed are duodenal ulcer and obstruction.

The methods of determination of pancreatic function (other than that of its internal secretion, which contains insulin, and which is discussed elsewhere (p. 315) are in an unsatisfactory condition. They center around tests for its enzymes in duodenal and gastric contents, as well as in feces, urine and blood.

The absence of pancreatic enzymes from duodenal juice may suggest pancreatitis (inflammation of the pancreas) or some abnormality of the pancreatic ducts.

Gastric contents usually show the presence of bile and pancreatic enzymes during digestion; and while pancreatic disease might be supposed to change the picture, little of a definite nature can be drawn.

The determination of fat in the feces, particularly when the disturbance may be traced both to the pancreas and the gall-bladder, is of value. Here large masses of undigested fat—due to lack of lipase and lack of efficient support from bile—are encountered. Sometimes as much as 70 per cent of the total dry matter is fat.

* How far we are from a complete understanding of the physiological rôle of some enzymes is brought out by a pertinent comment: "The rôle of the pancreatic trypsins is generally considered to be one of preparing the food proteins for a complete breakdown. If this be the only physiological function of the trypsins, it is difficult to understand why they exhibit such pronounced and narrowly limited specificities." [Bergmann, Fruton and Pollock, *Science*, **85**, 410 (1937).]

† Little and Caldwell, working with highly purified fractions, present evidence to show that the primary amino groups of the enzyme molecule (protein presumably) are essential to the activity of the enzyme.

The examination of the nitrogen content of the feces may sometimes be of value. Normally some 5 to 10 per cent of the nitrogen of the food fails to be absorbed and finds its way into the feces. Abnormally, in pancreatic disease, with an increased loss of effective proteolytic action, the amount of nitrogen in the feces may increase considerably. This is on the assumption that such an impairment does not involve absorptional facilities.

In obstruction of the pancreatic duct, the amylase (also called diastase) in the urine may be increased. In pancreatic disease, as in pancreatitis, the amount of amylase in the blood may increase considerably; this is often of marked diagnostic significance. This may also be true of serum lipase.

INTESTINAL JUICE

The small intestine itself secretes a juice which contains a number of enzymes of importance to digestion. Aminopeptidase and dipeptidase have already been mentioned (p. 225). Several additional proteolytic enzymes (prolinase, prolidase, etc.) are undoubtedly also present. In addition, there are enzymes which hydrolyze three of the disaccharides. Sucrase converts sucrose into glucose and fructose; maltase converts maltose into two molecules of glucose; lactase hydrolyzes lactose into galactose and glucose. Phosphatases—largely alkaline phosphatase?—split several of the compounds of phosphorus (nucleotides, hexosephosphate) yielding, as one of the products, inorganic phosphate. Enterokinase (p. 223), which is perhaps an enzyme, though not a digestive one, is also found in intestinal juice. The intestine also contains a lecithinase(?) which hydrolyzes lecithin into fatty acid, glycerol, phosphoric acid, and choline.

BILE

The formation of bile is one of the many activities of the liver The bile is stored in the gallbladder, which is attached to the liver During fasting, the bile accumulates in the gallbladder; and during digestion, especially during a meal rich in fats, bile leaves the bladder to enter the small intestine (see Figs. 61 and 62). The hormone cholecystokinin (p. 224) instigates the contraction of the gallbladder and, probably, the relaxation of the common duct sphincter.

Bile is alkaline in reaction (*p*H 7.8–8.6) and is composed of bile salts, bile pigments, lecithin, cholesterol, inorganic salts, etc. It is, in reality, both a secretion and an excretion, the secretory substances being represented by the bile salts and the excretory ones by the bile pigments, cholesterol, etc.*

Bile Salts.—The value of bile in digestion is largely due to these salts which are formed in the liver. They aid in the digestion of fat and fat-soluble vitamins, A, D, E and K. How this is brought about is now fairly clear. In the first place, mixed with fats, these bile salts lower the surface tension and increase the emulsification of fats, which

* There is evidence that protein in the food stimulates the formation of bile and bile salts.

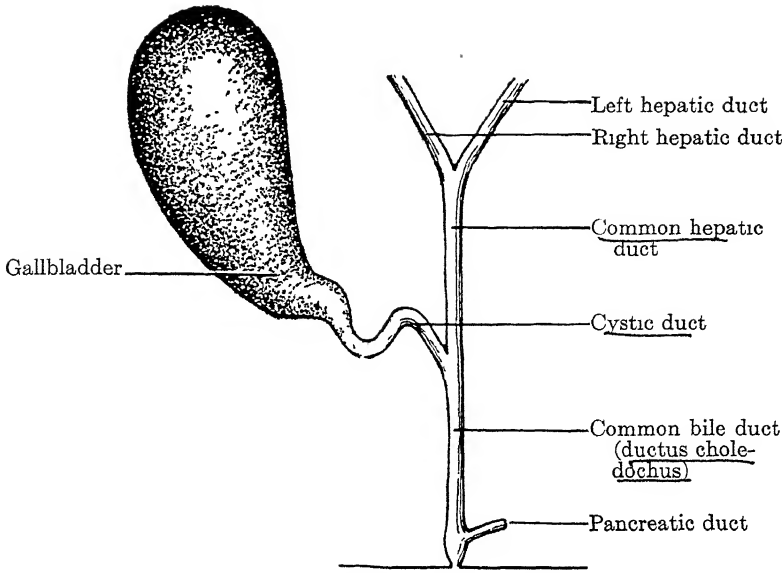


Fig. 61.—Diagram of bile ducts. (From Pitzman, *Fundamentals of Human Anatomy*, C. V. Mosby Co.)

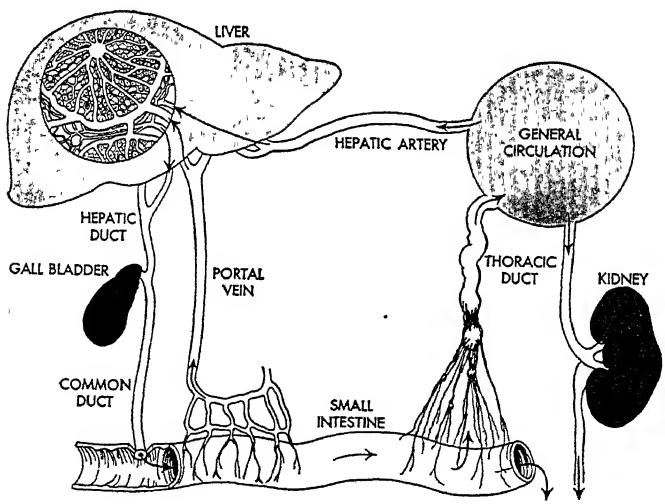


Fig. 62.—Circulation of bile. Precursors of bile acids are supplied by protein (glycine and taurine) and sterols (cholic acid). Formation of bile acids occurs in the polygonal liver cells. From these cells, the bile acids are secreted into bile canaliculi that lead to the intrahepatic ductal system.

Bile acids are absorbed along with fats. Most enter the portal circulation, are carried to the liver, and again secreted in the bile. Bile acids traverse this cycle repeatedly unless lost through the intestine, kidneys, or from biliary fistula. Some bile acids enter intestinal lymphatics, are carried to the general circulation, and return to the liver in the hepatic arteries. (*Therapeutic Notes*, by courtesy of Parke, Davis & Co.)

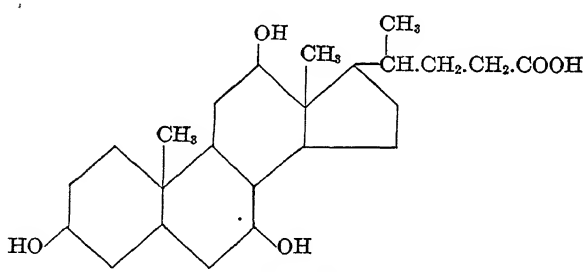
makes them more easily digested by lipase. In the second place, they combine with fatty acids, produced as a result of lipolytic action giving rise to a complex which is more soluble and more easily absorbed. The evidence points to the value of bile salts in promoting intestinal absorption of fats and fat-soluble vitamins on those occasions when there is a lack of bile in the intestine.

The bile salts are made up of sodium taurocholate and sodium glycocholate. The former is the sodium salt of taurocholic acid, a combination of taurine and cholic acid. Sodium glycocholate is the sodium salt of glycocholic acid, a combination of glycine and cholic acid.

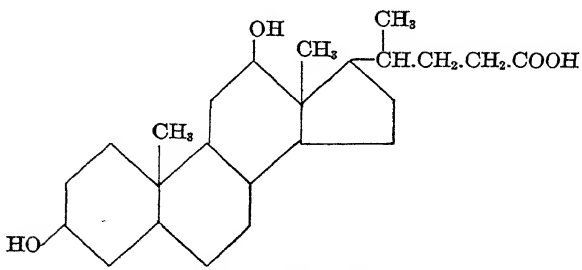
Taurine, $CH_2\,CH_2\,SO_3H$, or aminoethylsulfonic acid, is probably
NH_2
a derivative of cystine.

Cholic acid is related to cholesterol (p. 39) in structure; the carbon skeleton is similar, but the side chain is somewhat different, and the relationship of the first two rings, configurationally speaking, is also different. This makes it unlikely that the bile acids have their origin in cholesterol. However, Block and Rittenberg have shown that the administration of cholesterol containing heavy hydrogen to the dog gives rise to a cholic acid (isolated from the urine) which contains as much of the deuterium as the cholesterol in the blood and bile. This strengthens the biological relationship.

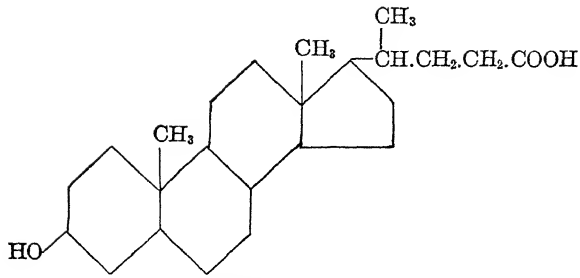
Besides cholic acid, several other closely related compounds are also found in bile, all of them, presumably, decomposition products of the "mother" sterol, and all of them showing a characteristic sterol configuration; examples are desoxycholic acid, and lithocholic acid.



Cholic acid.



Desoxycholic acid.



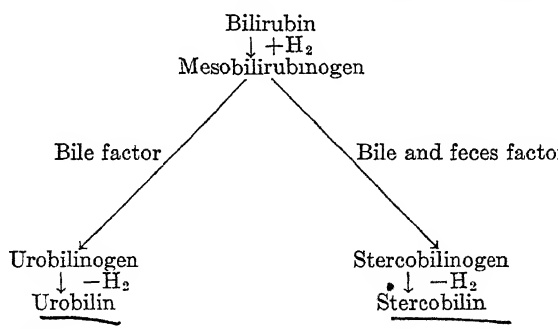
Lithocholic acid.

Bile acids are synthesized by the animal (from cholesterol?). The amount of these acids excreted is less than that formed. Nor do these acids accumulate in the animal body. By studying the fate of cholic acid in the guinea-pig, it can be shown that when the acid is injected intravenously it disappears from the body and is not eliminated in the excreta. However, the disappearance is due largely to decomposition within the cecum through the action of bacteria.

Bile Pigments.—These substances are decomposition products of hemoglobin (p. 258). They are oxidation products of the porphyrins (p. 258). The relationship may be shown as follows: hemoglobin → heme (p. 258) → protoporphyrin → biliverdin → bilirubin.

So far as is known, the bile pigments are purely excretory substances, although they are of value in identifying bile These pigments are biliverdin, bilirubin, etc., and give to the bile its characteristic color The stercobilin of the feces and the urobilin of the urine are related to the bilirubin of the bile.

This relationship may be shown in the form of a chart:



Tests for Bile.—One test depends upon the presence of the pigments. With an oxidizing reagent, such as nitric acid, a series of colored products are obtained (*Gmelin's test*). Another test depends upon the presence of the bile salts. With sucrose and concentrated sulfuric acid, a red color is obtained (*Pettenkofer's test*). This is probably not unlike the Molisch test for sugars (Chap. 2), involving the intermediate production of furfural.

Gallstones.—Gallstones (biliary calculus) are found largely in the bile ducts and in the gallbladder in pathological conditions, and may prevent bile from entering the intestines. They consist of cholesterol

or cholesterol mixed with the calcium salts of bilirubin, carbonate or phosphate.

The calculi are but one of a number of possibilities, often closely related. The calculi in the gallbladder and the bile ducts (cholelithiasis) is often associated with inflammation of the gallbladder (cholecystitis) and of bile passages (cholangitis).

Pure gallstones probably originate in the liver when the gallbladder is unable to handle the cholesterol reaching it. Mixed gallstones are often due to infection and inflammation of the gallbladder.

Diagnosis of gallbladder disease is often helped by an *x*-ray examination of the gallbladder and by the administration of tetraiodophenolphthalein.

Jaundice (icterus).—When bile pigments get into the blood the skin and secretions become yellow in color. In a common form of the disease, obstructive jaundice, this is due to complete or incomplete obstruction of the common duct (see Fig. 61). In another form, hemolytic jaundice, the disease is due to an extensive destruction of hemoglobin.

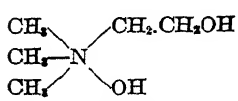
Various clinical tests are used. Among them are tests for bile pigments and bile salts in the urine; the icterus index, a test depending upon the increase in bilirubin which in turn changes the intensity of the yellow color of the blood plasma; test for urobilinogen in urine; and the van den Bergh test for bilirubin in plasma (or serum), which is said to distinguish obstructive jaundice (bilirubin which has passed through the liver) from hemolytic jaundice (in which the bilirubin has not passed through the liver).

PUTREFACTION

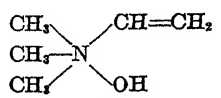
As we shall see presently (Chap. 12), most of the absorption of foodstuffs occurs in the small intestine. What is not absorbed passes on to the large intestine, where gradual loss of water occurs by absorption, and from where the products are evacuated finally as feces.

The normal stool is a mixture of water, undigested food, products of the digestive tract (bile pigments, enzymes, mucus), products of putrefaction (indole, skatole, fatty acids, gases, etc.), epithelial cells from the walls of the intestine, bacteria, etc.

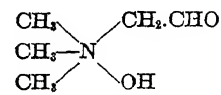
In the large intestine active bacterial action takes place. Gases (hydrogen, carbon dioxide, ammonia, hydrogen sulfide, methane), acids (acetic, lactic, butyric), various toxic substances (indole, skatole, phenol, etc.) are formed. The acids are largely products of the bacterial decomposition of carbohydrates. Some special substances, such as choline, neurine, and muscarine, have their source in lecithin. The most characteristic group of substances are derived from the proteins.



Choline.

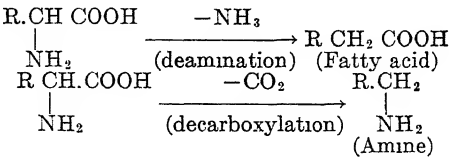


Neurine.



Muscarine.

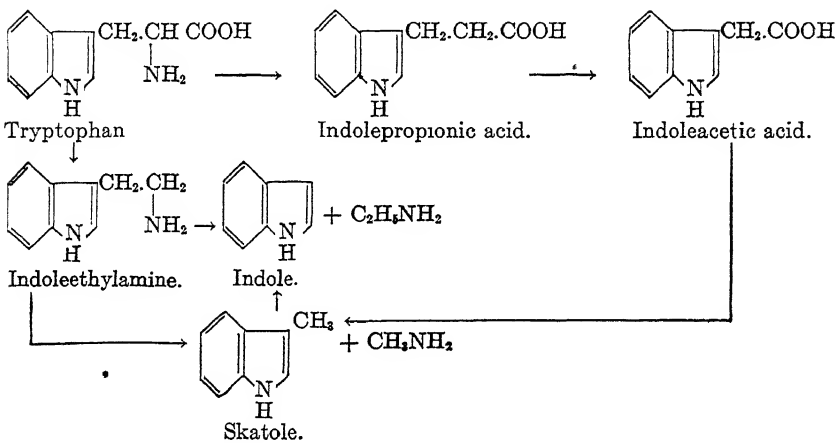
After a preliminary hydrolysis into their respective amino acids, the latter undergo a series of reactions involving deamination and decarboxylation. These reactions can be illustrated as follows:



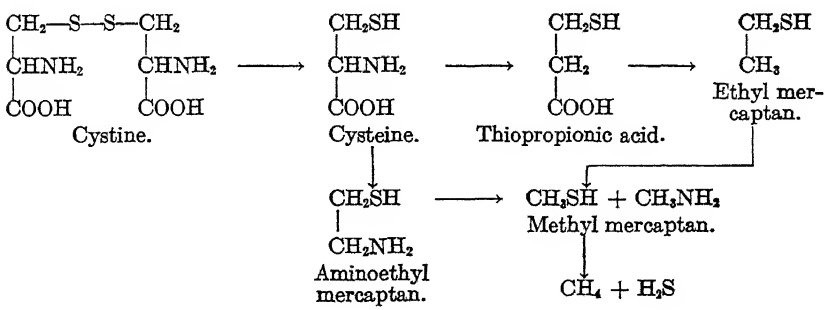
giving rise in the amines to some highly toxic substances.

To illustrate the process, we will select a number of amino acids which produce characteristic products

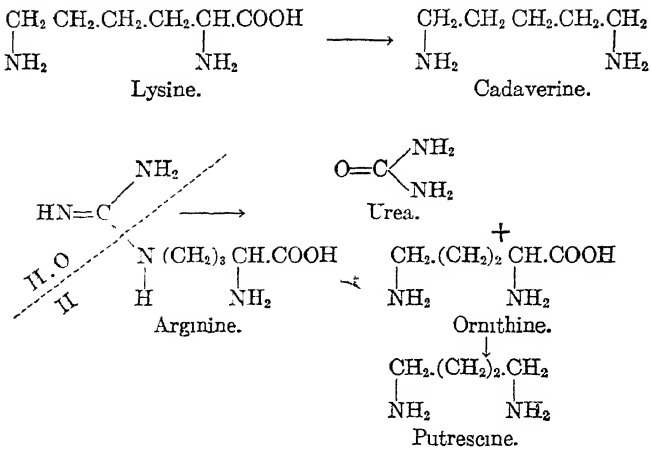
Tryptophan forms, among others, indole and skatole, substances partially responsible for the odor of feces:



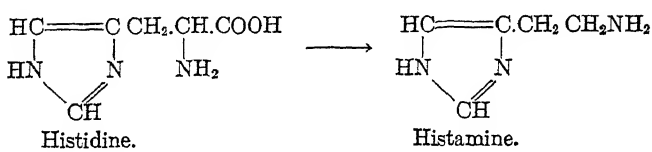
Mercaptans are formed from the sulfur-containing amino acid cystine (and methionine?):



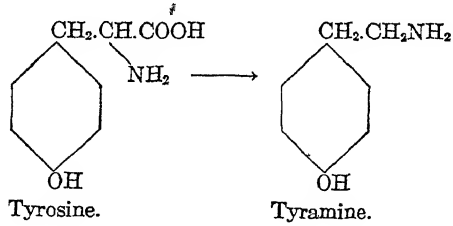
The so-called "ptomaines," substances obtained from putrefying flesh, may be formed by the decarboxylation of lysine and arginine, giving rise to cadaverine and putrescine, respectively:



Histamine, obtained from histidine by decarboxylation, is a highly toxic substance when injected, and some have claimed it to be identical with the gastrin of the stomach:



Tyramine, obtained from tyrosine, is somewhat similar in its reaction to adrenaline in raising blood pressure:



Intestinal Flora.—Probably 25 per cent of the dried feces represents bacteria, mostly of the nonpathogenic variety. Bacterial decomposition of whatever foodstuffs remain in the large intestine is of particular importance to herbivora, for in this way much of their food is utilized. The colon bacillus is the commonest organism found in man, and we have seen how the putrefactive products—toxic substances—are thereby produced. An organism present in much smaller quantity belongs to the aciduric group. Such bacteria produce lactic acid from carbohydrates. The addition of dextrin or lactose to the diet brings about a greater production of the aciduric group, and the acid produced as a result of their metabolic activity tends to establish a medium which is unfavorable to the colon bacillus.

The more favorable medium, containing an optimum of the aciduric

organisms, is also supported by a well-balanced inorganic diet, particularly by the addition of both calcium and phosphorus.

Despite the fact that with fruits and vegetables, for example, we ingest cellulose, there is little evidence for any digestion of it by man (though it facilitates proper digestion). Herbivorous animals and insects do utilize cellulose. This is due to the action of various microorganisms in their digestive tracts.

Normally the stool contains water, undigested foods, material which cannot be digested (vegetable cells and fibers), products of the digestive tract (changed bile pigments, enzymes, mucus), products obtained through bacterial decomposition (indole, skatole, etc.) and bacteria, mainly harmless. Under abnormal conditions, we may find blood, pathogenic bacteria, etc.

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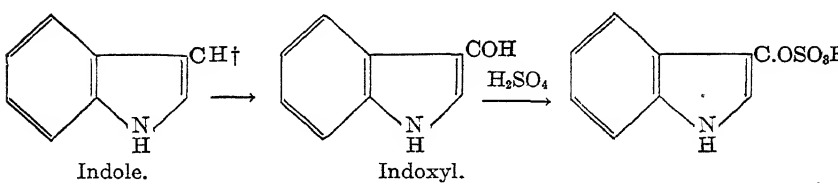
CHAPTER 11

DETOXICATION

THE toxic substances produced in the large intestine and discussed in the previous chapter are largely eliminated in the stool; those which are not, are detoxified—that is, absorbed, combined in the liver or kidney with perhaps sulfuric or glucuronic acid, and then eliminated through the kidneys into the urine.

In a general way, it may be said that, in the attempt to detoxify a substance introduced into the body, several methods are employed: toxic material may be oxidized; or, the toxic substance may combine with glucuronic acid, sulfuric acid, one of the amino acids, etc., and thereby bring about a detoxified product.*

In the last chapter we discussed the production of indole from tryptophan, a bacterial change brought about in the large intestine. Much of the indole is eliminated in the feces. Some of it is absorbed, partially oxidized, and combined with sulfuric acid to form indican, which is eliminated in the urine.



Indole. Indoxyl. Indican.

The amount of indican in the urine may be indicative of the extent of putrefaction in the large intestine.

That "detoxication" is not always a conversion of a toxic to a non-toxic body is made clear by one or two examples. For instance, the female sex hormone is eliminated (to some extent, at least) as a glycuronate and the male sex hormone (also to some extent) as the sulfate. Obviously, neither of these hormones, of importance to the body, can be considered as toxic substances. Again, as Quick points out, the formation of taurocholic acid from cholic acid and glycine—a normal metabolic process—cannot, in reality, be distinguished from a

* While "detoxication" connotes a lessening of toxicity, sometimes the detoxified product may still prove quite toxic.

† The CH, shown here for convenience, is really part of the ring structure

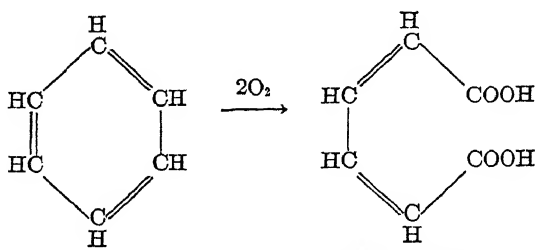
"detoxication" involving glycine in other reactions (see, for example, p. 239).

Since the excreted product is often the more acidic, Quick advances the theory that the substance which is "detoxified" by the body, because it is less acidic, would be eliminated with difficulty, and that when "detoxicated," the kidneys eliminate this coupled product more readily.

The changes which some substances undergo in the body will now be discussed. Such changes are usually studied in experiments on animals. The usual procedure is to feed or inject the substance under investigation into the animal (dog, rabbit, etc.), collect the urine over a given period of time, and recover from it changed and unchanged material. The practical difficulties are often very great; and much of the information at present is in a very incomplete state.

The tissue slice technic developed by Warburg is also employed. Here pieces of tissue, properly buffered, are mixed in a Warburg apparatus with the substance under examination, and an attempt is made to isolate any products formed. Of course, the objection may be raised that an isolated part of an organ is hardly the equivalent of a real *in vivo* experiment.

Oxidation.—In a general way, it may be said that aliphatic compounds are more easily oxidized than aromatic ones. It is true that this does not apply to α-amino acids attached to aromatic nuclei. These are, of course, foods and not poisonous products. For example, so far as we know, phenylalanine and tyrosine, both containing the benzene nucleus, are quite readily oxidized in the animal organism. On the other hand, the fate of benzene itself is still largely a matter of dispute. Many years ago, Jaffe claimed to have isolated muconic acid as a result of feeding benzene; this has been confirmed by Drummond and others.



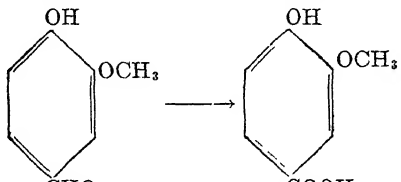
Muconic acid.

However, some of this benzene is converted into phenol by both man and the dog. Some of the phenol, in turn, is excreted in conjugated form, partly with sulfuric acid and partly with glucuronic acid.

Toluene and ethylbenzene are oxidized to some extent to benzoic acid. With *m*-xylene, $C_6H_4(CH_3)_2$, containing two methyl groups, one methyl group is oxidized to a carboxylic acid group. This is also true of mesitylene, the symmetrical trimethylbenzene, $C_6H_3(CH_3)_3$, which, when ingested, has but one of its CH_3 groups converted to COOH.

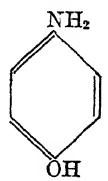
Primary aromatic alcohols are often oxidized to the corresponding carboxylic acids. For example, benzyl alcohol, $C_6H_5.CH_2.OH$, is converted to benzoic acid, C_6H_5COOH, and phenylethyl alcohol, $C_6H_5.CH_2\ CH_2\ OH$, to phenylacetic acid, $C_6H_5.CH_2.COOH$.

Aromatic aldehydes are usually oxidized to the corresponding carboxylic acids. Benzaldehyde, $C_6H_5.CHO$, for example, is converted into benzoic acid. Vanillin is oxidized to vanillic acid.



Vanillin. Vanillic acid.

Whereas aliphatic amines—like aliphatic compounds, in general—are apt to be destroyed by the body, aromatic amines are sometimes converted to carboxylic acids. Benzylamine, $C_6H_5.CH_2NH_2$ is oxidized to benzoic acid. Where, however, the NH_2 group is attached to the benzene nucleus, the results are different. For example, aniline, $C_6H_5.NH_2$, itself, is oxidized to *p*-aminophenol,

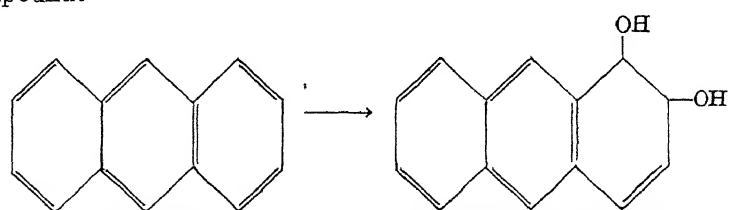


p-Aminophenol.

though it is finally excreted in combination with sulfuric acid and possibly glucuronic acid.

Acetanilide, $C_6H_5.NH.CO.CH_3$, is oxidized to *p*-aminophenol in the rabbit, and to *p*-acetylaminophenol, $HO.C_6H_4.NH.CO.CH_3$, in man.

The interest in sex hormones and carcinogenic substances related to them (Chap. 24), has led to a metabolic study of polycyclic derivatives. Anthracene, for example, is partially oxidized to a dihydroxy compound:

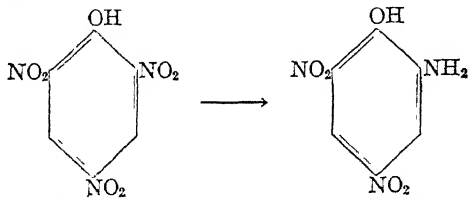


Anthracene. 1,2-Dihydroxy-1,2-dihydro-anthracene.

although some of it also combines with glucuronic acid, and some with cysteine.

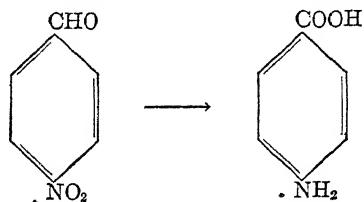
The carcinogenic compound, 1,2,5,6-dibenzanthracene (p. 521) is converted into a dihydroxy compound

Reduction.—Though less common than oxidation, reduction of substances in the animal body does occur. (We exclude here reactions in the large intestine, where reducing bacteria are very active.) A classical example of this type of reaction is the conversion of picric acid into picramic acid.



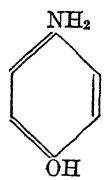
Picric acid. Picramic acid.

Sometimes a simultaneous reduction and oxidation within the same compound will occur, for example, when rabbits are fed *p*-nitrobenzaldehyde they excrete appreciable quantities of *p*-aminobenzoic acid:



p-Nitrobenzaldehyde. *p*-Aminobenzoic acid.

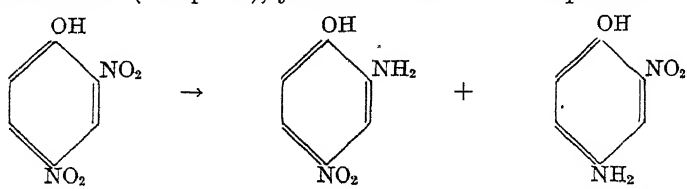
Nitro compounds, as a fairly general rule, are converted to the corresponding amino compounds. The simplest aromatic nitro derivative nitrobenzene, $C_6H_5NO_2$, reacts similarly to *p*-nitrobenzaldehyde, because reduction and oxidation occur, to give rise to *p*-aminophenol:



p-Aminophenol.

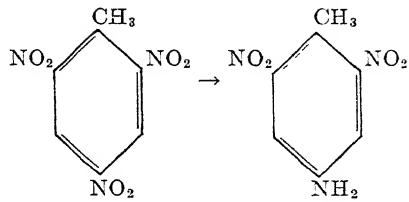
It is interesting to note that both aniline and *p*-nitrophenol, HO.$C_6H_4NO_2$, also form *p*-aminophenol.

2,4-Dinitrophenol, one of a number of nitrophenols which affect basal metabolism (Chap. 20), yields a mixture of compounds.



2,4-Dinitrophenol. 2-Amino-4-nitrophenol. 2-Nitro-4 aminophenol.

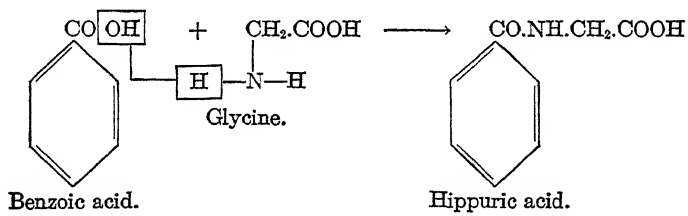
2,4,6-Trinitrotoluene (T.N.T.), is partially reduced to 2,6-dinitro-4-aminotoluene:



and also partially excreted as the glucuronide (p. 242).

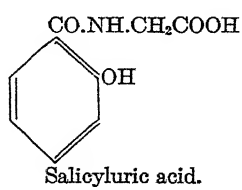
Conjugation.—Where oxidation fails, conjugation becomes an alternate (or added) procedure. (As we have seen, a substance like anthracene can be partly oxidized and partly conjugated.) Conjugation involves the combination of the toxic product with some substance available to the body to form a detoxified product which is then eliminated. The substances known to be used by the body for detoxifying purposes are glycine, glutamine, ornithine, cysteine, sulfuric acid, glucuronic acid, acetic acid, and the methyl group.

Glycine.—This amino acid seems to attach itself to acids in particular. The well-known example is the production of hippuric acid by feeding benzoic acid:



However, combination of acids with glucuronic acid is also common.

It might be expected that *o*-hydroxybenzoic acid, which is the compound of pharmacological importance known as salicylic acid, would also be detoxified in the body by combining with glycine. It is true that a small quantity of salicyluric acid



(the combination of salicylic acid and glycine) has been isolated, but this quantity can hardly represent the main metabolic product

p-Hydroxybenzoic acid is excreted partly uncombined and partly conjugated with glycine.

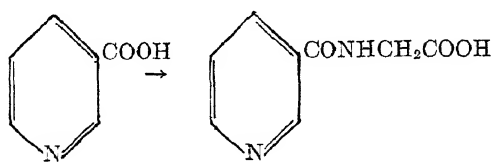
In dealing with detoxifying agents, it should be pointed out at the very outset that because the horse forms hippuric acid when it is fed

benzoic acid, one cannot necessarily conclude that hippuric acid will always be formed, irrespective of the animal used. As a matter of fact, in this instance, with one exception, every vertebrate so far tried forms hippuric acid. The one exception is the fowl, which uses ornithine in the place of glycine (p. 241).

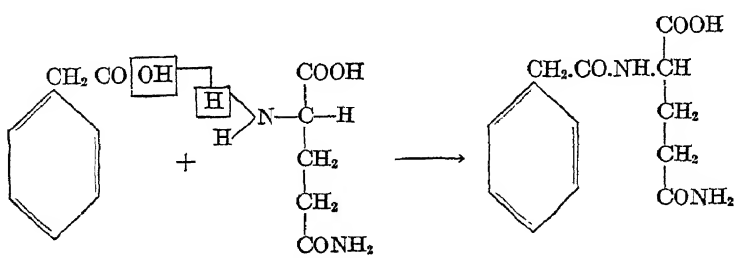
Just as deuterium has proved of value in the metabolism of fats (Chap. 17), the nitrogen isotope is now being introduced to study the metabolism of nitrogenous compounds, including proteins and amino acids (Schoenheimer and Rittenberg). Urey, who first prepared deuterium, has succeeded in concentrating the nitrogen isotope. The ordinary nitrogen has an atomic weight of 14 (N^{14}); the isotope has an atomic weight of 15 (N^{15}). Urey prepared a sample of ammonia enriched with N^{15}. The normal ratio, N^{14}/N^{15} is 266, whereas in the enriched ammonia the ratio is 160 Starting with this enriched ammonia, glycine and hippuric acid were prepared. The isotope analyses were carried out with a mass spectrometer.

The formation of hippuric acid in the body was studied. It was shown that hippuric acid can be absorbed from the intestinal tract without being hydrolyzed. Furthermore, glycine may be utilized directly for hippuric acid formation.

Nicotinic acid (p. 159) is partly conjugated with glycine in the body to form nicotinuric acid:



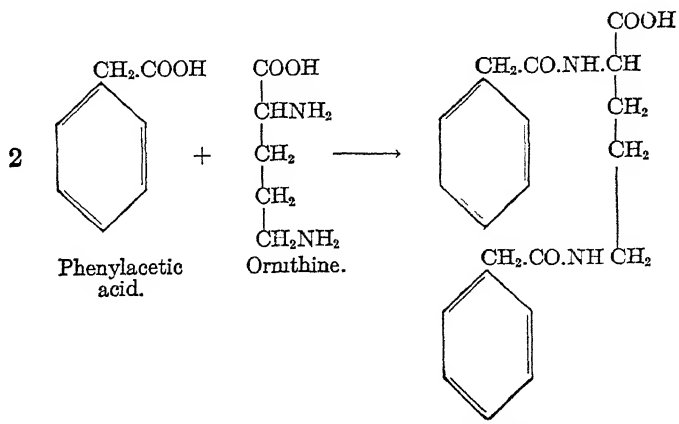
Glutamine.—This amide of glutamic acid is an active detoxifying agent in human beings only and in the chimpanzee; at least, in so far as the record up to the present is concerned. The ingestion of phenylacetic acid by a human being will cause the production of phenylacetyl glutamine, a product which can be isolated from the urine:



Phenylacetic acid. Glutamine. Phenylacetylglutamine.

As illustrating once again the difference in behavior depending upon the animal used, in the fowl, phenylacetic acid combines with ornithine, and in most other animals, phenylacetic acid combines partly with glycine and partly with glucuronic acid.

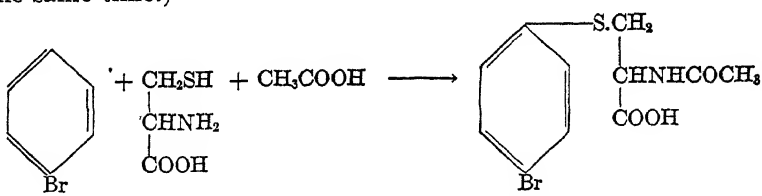
Ornithine.—As has already been indicated, the fowl is the one animal that utilizes ornithine for detoxifying purposes. With phenylacetic acid the reaction is as follows:



Phenylacetic acid. Ornithine.

Diphenylacetylornithine.

Cysteine.—The feeding of bromobenzene to dogs results in the formation of a mercapturic acid; which means that cysteine is used for detoxifying purposes. (The amino group of the cysteine is acetylated at the same time.)

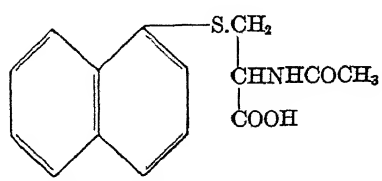


Bromobenzene. Cysteine. *p*-Bromophenylmercapturic acid.

Some of the bromobenzene is oxidized to *p*-bromophenol, and the latter is excreted partly in combination with sulfuric acid and partly in combination with glucuronic acid.

Chlorobenzene, iodobenzene and fluorobenzene are converted into the corresponding mercapturic acid derivatives.

An important contribution to cysteine as a detoxifying agent we owe to Bourne and Young. They discovered that in rabbits naphthalene is partially converted into its mercapturic acid:*



Naphthylmercapturic acid.

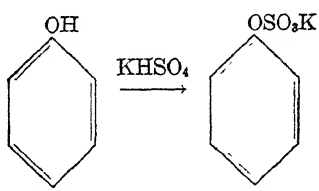
* When naphthalene is repeatedly administered to rabbits, the crystalline

There is evidence at hand that anthracene is detoxicated in a similar manner; and some claim this to be true also of phenanthrene.

Small quantities of phenylmercapturic acid have been isolated by Young after administering benzene to rats.

The evidence for the formation of mercapturic acid with benzene and phenanthrene is an increased neutral sulfur excretion (p. 470) when they are administered. Further evidence is afforded by the fact that on certain synthetic diets the growth of rats is inhibited when phenanthrene (or naphthalene or bromobenzene) is added to the diet. Probably the explanation is that phenanthrene attaches itself to cystine (or other sulfur-containing compounds), thereby making the latter unavailable to the animal. If phenanthrene and cystine (or methionine) are simultaneously added to the diet, no deficiency occurs.

Ethereal Sulfates.—The well-known example of the formation of indican from indole (p. 235) is an instance of this type of detoxication. Phenol behaves similarly:



Phenol. Phenol potassium sulfate.

In general, hydroxy compounds have a tendency to form such sulfate combinations; although, on the whole, glucuronates are more common.

Not only is part of the phenol conjugated but part of it is oxidized and part of it excreted unchanged. (In many of these detoxication experiments one finds very appreciable quantities of the ingested material in the urine.)

In the case of a methyl substituted phenol, such as *p*-cresol, $HO.C_6H_4.CH_3$, this substance, when ingested, is first converted to *p*-hydroxybenzoic acid, $HO.C_6H_4.COOH$, and then probably converted to the corresponding sulfate or glucuronate salts.

To some extent, androsterone, a male sex hormone, is eliminated in sulfate form.

It would seem, from several experiments, that the administration of adrenaline markedly increases the elimination of ethereal sulfates. This suggests that the inactivation of adrenaline in the body is not necessarily accomplished by, say, oxidative destruction, but by coupling it with sulfuric acid.

Glucuronic Acid.—Combinations with this acid are extremely common. Benzoic acid combines not only with glycine but also with glucuronic acid to form 1-benzoylglucuronic acid:

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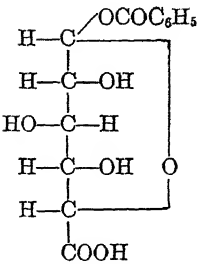
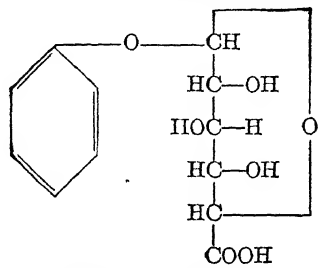
lens of the eye undergoes degeneration—a process which resembles that observed in human senile cataract.



1-Benzoylglucuronic acid.

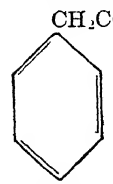
The same is true of phenol: it also combines with glucuronic acid (as well as with sulfuric acid) to form phenolglucuronic acid:



Phenolglucuronic acid.

The combination of benzoic acid with glucuronic acid can be shown in the pig, the dog, the sheep and in humans. In the last of these some 5 per cent is eliminated as the glucuronide. The maximum excretion occurs in the first three hours, and detoxication is complete in nine to fifteen hours, depending on the amount of benzoic acid ingested.

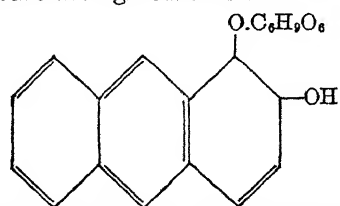
Phenylacetic acid, the first homologue of benzoic acid is also eliminated as a glucuronide to the extent of about 7.5 per cent.



Phenylacetic acid.

The indicator (and drug) phenolphthalein is detoxified, to some extent at least, as its glucuronide.

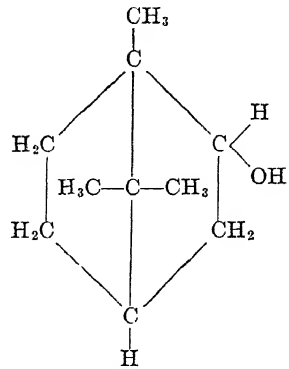
Anthracene is not only partially oxidized, as we have seen, but combines to some extent with cysteine to form a mercapturic acid, and combines to some extent with glucuronic acid to form a glucuronide:



1,2-Dihydroxy-1,2-dihydroanthracene glucuronide.

Marrian has shown that the female sex hormones are eliminated, to a certain extent, as glucuronates (Chap. 24).

Borneol, the secondary alcohol obtained from camphor when the



Borneol

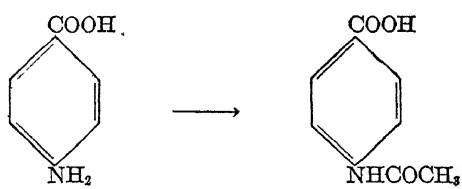
latter is reduced, combines almost exclusively with glucuronic acid In humans, the ingestion of borneol results in the excretion in twenty-four hours of some 90 per cent or more in the form of its glucuronide. The maximum excretion occurs in from three to six hours, and the detoxication is complete in about fifteen hours.

The combination of borneol (and menthol or phenol) with glucuronic acid may also be shown by using surviving liver slices in a saline medium (at *p*H 7.4), to which borneol is added, and shaking the mixture in a Warburg apparatus.

Sulfapyridine (p. 284) is eliminated, to some extent, as a glucuronide of a hydroxysulfapyridine.*

Glycoside (p. 14) formation in plants might correspond to the formation of glucuronides in animals, in the sense that, as a rule, less toxic substances are produced.†

Acetic Acid.—This acid is used by the organism for the detoxication of amino groups. One such example has already been given in the formation of the mercapturic acids (p. 241). Another well-known example is the acetylation of *p*-aminobenzoic acid:



p-Aminobenzoic acid. *p*-Acetylaminobenzoic acid.

* Lewis has been able to show the conversion of an *aliphatic* fatty acid to its glucuronide by the administration (to rabbits) of trimethylacetic acid and tertiary butylacetic acid.

† An enzyme, β-glycuronidase, is active in the "coupling" of glucuronic acid with various substances.

Lewis has presented evidence to show that liver glycogen (rather than body glucose) is a source of glucuronic acid in the body.

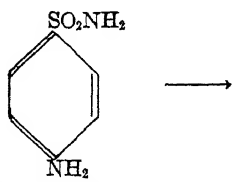
So far, acetylation has been observed only in amino compounds.*

Insulin markedly increases the output of *p*-acetylaminobenzoic acid. That this is a result of a stimulating effect on carbohydrate metabolism seems probable.

Reduced glutathione alone has no effect upon the acetylation process But the simultaneous injections of insulin and glutathione very definitely inhibit the output of *p*-acetylaminobenzoic acid. Such a result is probably due to the inactivation of insulin by glutathione.

Approximately 25 per cent of the *p*-aminobenzoic acid can be accounted for as the acetylated product. Some of the acid is also eliminated in the form of the glucuronate.

Sulfanilamide, a compound which has come into much prominence, is largely excreted in the form of the acetylated derivative:



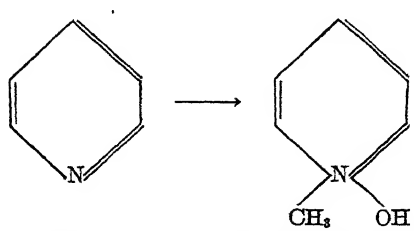
Sulfanilamide. (*p*-aminobenzene-sulfonamide).

p-Acetylaminobenzene-sulfonamide.†

From 50 to 90 per cent of the sulfanilamide administered can be accounted for by the acetylated and the free drug which is eliminated in the urine. However, there is an increase in the output of ethereal sulfate (p. 469); and this suggests that some of the sulfanilamide is changed in the body to a hydroxy compound, possibly a phenol (*p*-aminophenol?), which is then eliminated in the combined form with sulfate.

This reaction with sulfanilamide, so far as it has been studied, applies to man and to the rabbit but not to the dog.

Methylation.—It was supposed until recently that this type of detoxication was not common. One of the few examples quoted was the conversion of pyridine to a methyl derivative:



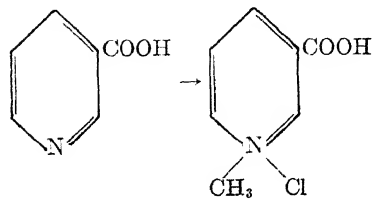
Pyridine. Methyl hydroxypyridine.

* This is not true of the *normal* metabolic process. For example, acetylcholine (p. 537) is constantly formed in nervous tissue.

† This acetylated product is, if anything, more toxic than sulfanilamide. Such a reaction is difficult to formulate as a "detoxifying combination."

However, it has now been shown that methylation in the body occurs very frequently. Both choline (p. 35) and methionine (p. 54), for example, can supply their methyl groups for various needs (p. 337).

In this connection it may be mentioned that a closely related compound to pyridine, namely nicotinic acid (p. 159), is methylated to some extent in the body, forming trigonelline chloride:



The methyl group required for this reaction is probably derived from methionine.

The Liver and Detoxication.—In the main, the liver seems to be the seat for the detoxicating process, although experiments are not wanting to prove that often the kidney and other organs function also.

Using the tissue slice technic, Borsook has found that in the guinea-pig, rabbit and rat, hippuric acid synthesis (conjugation of benzoic acid and glycine) can occur in both kidney and the liver; whereas in the dog it occurs in the kidney but not the liver.

It is interesting to note that when the tissue cell structure is destroyed by maceration or poisoned with toluene or cyanide, no synthesis takes place.

Detoxicating reactions have been suggested at various times for testing organic function. They have met with varying success. See, for example, Cantarow and Trumper, *Clinical Biochemistry* (1945), 454.

Glutathione.—Aside from a possible rôle which glutathione plays in oxidative mechanisms (p. 400), the suggestion has been advanced that it may be of importance as a detoxifying agent. It is, to say the least, extremely suggestive that this polypeptide consists of three amino acids (glycine, cysteine, glutamic acid), each one of which is known to act as a detoxifying agent. Evidence in favor of this view has been presented.

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Borsook and Dubnoff, *J. Biol. Chem.*, **132**, 307 (1940), discuss the biological synthesis of hippuric acid *in vitro*.

For the acetylation of *p*-aminobenzoic acid see Harrow, Mazur and Sherwin, *J Biol Chem*, **102,** 35 (1933).

Illustrations of cysteine as a detoxifying agent may be found in the articles by Young, *Biochem. J*, **28,** 803 (1934) (the fate of naphthalene in the body), and West, *Proc Soc Exp Biol Med.*, **43,** 373 (1940) (the detoxication of diphenyl through a sulfur mechanism).

Stekol has published a series of papers on mercapturic acid synthesis. See, for example, *J Biol. Chem.*, **122,** 333 (1938); **127,** 131 (1939).

References to glucuronic acid as a detoxicating agent are many. See, for example, Di Somma, *J. Biol. Chem*, **133,** 277 (1940) (phenolphthalein); Kensler, Young and Rhoads, *Proc. Soc. Exp Biol. Med*, **48,** 22 (1941) (effect of dimethylaminoazobenzene—butter yellow—on glucuronic acid production); Wagreich, Kamin and Harrow, *Ibid.*, **43,** 468 (1940); Wagreich, Abrams and Harrow, *Ibid.*, **45,** 46 (1940); Wagreich, Bernstein, Pader and Harrow, *Ibid*, **46,** 582 (1941).

The source of glucuronic acid in the body is discussed by Dziewiatkowski and Lewis, *J. Biol. Chem*, **153,** 49 (1944)

The estimation of glucuronic acid in the urine (and its application) is described by Deichmann and Thomas, *J. Industrial Hygiene and Toxicology*, **25,** 286 (1943).

What appears to be the first case of the conjugation of glucuronic acid with an aliphatic fatty acid is recorded by Dziewiatkowski and Lewis, *J. Biol. Chem.*, **158,** 77 (1945).

That sulfapyridine is partially eliminated as a glucuronide is shown by Scudi, *Proc Soc. Exp. Biol. Med.*, **55,** 197 (1944).

For the fate of sulfanilamide in the body, see a series of articles by Shelwell and Williams, *Bioch. J.*, **34,** 528 (1940); Thorpe, Williams and Shelswell, *Ibid*, **35,** 52 (1941); Thorpe and Williams, *Ibid*, **35,** 61 (1941); Sammons, Shelswell and Williams, *Ibid*, **35,** 557 (1941); Williams, *Ibid*, **35,** 1169 (1941).

A study of the fate in the body of the carcinogenic compound, dibenzanthracene, is described by Boyland, Levi, Mawson and Roe, *Biochem. J.*, **35,** 184 (1941).

Reference to the possibilities of glutathione as a detoxifying agent will be found in the paper by Harrow, Chamelin and Mazur, *Proc. Soc. Exp. Biol. Med.*, **37,** 271 (1937).

For the fate of fluorobenzene in the body, see Young and Zbarsky, *J. Biol. Chem.*, **154,** 389 (1944).

Zbarsky and Young, *J. Biol. Chem.*, **151,** 487 (1943) show that small quantities of benzene are converted into the mercapturic acid derivative in the body.

Deichmann, *Arch. Biochem.*, **3,** 345 (1944), discusses several angles of the detoxication of phenol. See, also, DeMeio and Arnolt, *J. Biol. Chem*, **156,** 577 (1944).

Fishman and Cohn, *J. Biol. Chem.*, **148,** 619 (1943), and Doisy, Jr. and Westerfeld, *Ibid*, **149,** 229 (1943), speculate concerning the mechanism of the acetylating process.

A study of the fate in the body of several halogenated phenols used in disinfection is presented by Zondek and Shapiro, *Biochem. J.*, **37,** 592 (1943).

A very thorough study of the fate of T.N.T. in the body was undertaken by Channon, Mills and Williams, *Biochem. J.*, **38,** 70 (1944).

A critical evaluation of various liver functional tests is presented by Mateer, Baltz, Marion and MacMillan, *J. Am. Med. Assoc.*, **121,** 723 (1943); and Steigmann, Topper and Meyer, *Ibid*, **122,** 279 (1943).

CHAPTER 12

ABSORPTION

BEFORE being absorbed, foods must be in a relatively simple (chemically speaking), soluble form. The action of the digestive juices converts much of the foodstuffs into amino acids, hexoses (glucose, fructose, and galactose), glycerol and fatty acids. If pentosans and mannans are present, pentoses and mannose may be formed. Some pentose is also formed from animal nucleic acid The cellulose in the diet remains unchanged and passes into the large intestine, acting as

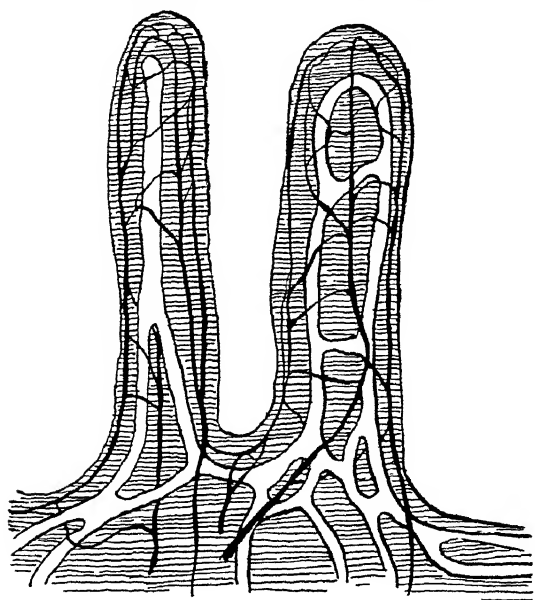


Fig. 63.—Diagram of villi of human intestine. Lacteals are white and blood vessels are dark. (From Christian, *Anatomy for Nurses*, C. V. Mosby Co)

"roughage." Some bacterial decomposition of cellulose probably does take place. This is particularly true of herbivorous animals.

In general, absorption through the stomach wall is slight. By ıcluding the pylorus, 99 per cent of sugar can be recovered from the tomach several hours after feeding. Not even water is absorbed. n a particular experiment on a dog, with a fistula in the duodenum ust beyond the pylorus, of 500 cc. of water offered by mouth, 495 cc. ppeared through the duodenal fistula in twenty-five minutes. Alcohol, ın the other hand, seems to be absorbed quite readily.

The absorption of food—which includes much, but not all of the water—takes place most readily through the walls of the small intestine. The tube is some 25 feet long, and its surface is considerably

multiplied by the villi, finger-like projections some 1 mm. in length (Fig. 63). Using a fistula at the end of the small intestine, it has been shown that, on the average, some 90 per cent of the protein is absorbed. This applies equally well to the carbohydrates and fats. Water is absorbed here too, but its loss is apparently made up by diffusion of liquid into the intestine, for the food at the ileocecal valve is still very fluid.

In the large intestine there is considerable absorption of water, resulting in a residue which eventually appears as feces. As we have already seen, bacterial action resulting in putrefactive processes is here very pronounced.

For the general plan of absorption, see Fig. 64.

Absorption of Carbohydrates.—The villi of the small intestine contain blood vessels, nerves, and lymphatics, and it is through these villi that absorption takes place. There are two possible paths. One is absorption through the capillaries, the absorbed material passing via the portal system into the liver before entering the general circulation. The other is absorption through the lacteals, the material then passing via the lymph into the thoracic duct and finally into the blood. The weight of evidence is that the absorption of carbohydrates takes place through the capillaries of the villi. These carbohydrates in order to be absorbed must be in the form of hexoses—glucose, fructose, galactose, and mannose. Pentoses, if present, are also absorbed. Furthermore, combination with phosphoric acid to form hexose phosphate possibly takes place before absorption. The evidence for this view is that two substances which prevent the formation of such phosphates, the glucoside phloridzin and iodoacetic acid, are precisely the two substances which prevent the absorption of sugars in the small intestine.

There is a difference in the rate of absorption of different hexoses, indicating a selective action on the part of the intestinal mucosa. The order is galactose > glucose > fructose > mannose. Another somewhat puzzling feature in terms of a simple physiochemical conception is that the rate of absorption remains the same over a comparatively long period—in fact, until most of the sugar is absorbed.

In this connection, it is of interest to point out that normally glucose is more rapidly absorbed than a pentose. However, if a dead mucous membrane is used, or if the membrane is first poisoned with iodoacetic acid, the absorption is in the reverse order: first the smaller pentose molecule, and then the hexose.

The hexoses are changed largely to glycogen in the liver and stored there as such until needed by the body. Preliminary phosphate combinations are necessary. (The further fate of carbohydrates is discussed under the metabolism of these substances, Chap. 16.) It is not clear at present whether, prior to the synthetic formation of glycogen, hexoses other than glucose are first transformed into the latter, or whether each hexose is polymerized into glycogen. In any case, when glycogen itself is hydrolyzed, glucose and glucose alone is the product. Nor is it clear as to just what happens to such pentoses as xylose and

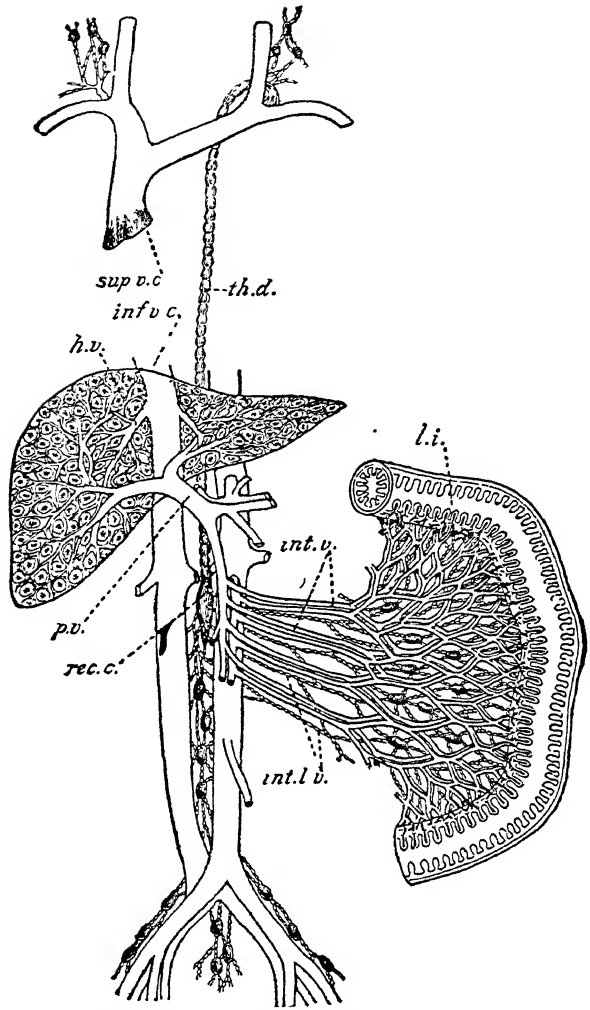


Fig. 64 —Diagram Showing the Routes by which the Absorbed Foods Reach the Blood of the General Circulation (*G Bachman*). *l. i*, Loop of small intestine; *int. v.*, intestinal veins converging to form in part, *p. v.*, the portal vein, which enters the liver and by repeated branchings assists in the formation of the hepatic capillary plexus; *h. v.*, the hepatic veins carrying blood from the liver and discharging it into, *inf. v. c.*, the inferior vena cava, *int. l. v.*, the intestinal lymph vessels converging to discharge their contents, chyle, into *rec. c.* the receptaculum chyli, the lower expanded part of the thoracic duct, *th. d.*, the thoracic duct discharging lymph and chyle into the blood at the junction of the internal jugular and subclavian veins; *sup. v. c.*, the superior vena cava.—(*From Brubaker's Textbook of Physiology.*)

arabinose when they are absorbed. The weight of evidence at present is that they are not glycogen-formers and that they are not utilized by the body.

Absorption of Fats.—The fats are not absorbed as such. They are first hydrolyzed by the lipase in the intestine into glycerol and fatty acids. The glycerol is quite soluble and is easily absorbed. This is not true of the fatty acids, which are quite insoluble in the aqueous medium. For a time a favorite theory was to suppose that the fatty acids were largely converted into soaps, and that the latter, being soluble, were absorbed. Unfortunately for this theory, the formation of such soaps would require the *p*H of the intestinal contents to be around 9; actually, it is often less than 7. More recent evidence points to an actual combination of the bile salts with the fatty acids, producing products which are soluble and which can be absorbed even at a *p*H below 7.*

How important the bile is in the process involving fat absorption becomes apparent when the bile duct is occluded. Under such conditions, relatively large quantities of undigested fat appear in the feces.

The glycerol and the complex of fatty acids are absorbed through the lacteals and are resynthesized into fat. Combination with phosphoric acid (phosphorylation) is a probable preliminary to actual resynthesis. There is some evidence that this resynthesized fat is not quite the same as the original fat in the food. In any case, this resynthesized fat passes into the lymph and finally into the venous circulation via the thoracic duct.

During a meal rich in fat, the lymphatics of the mesentery are filled with fat in a finely emulsified form; this also becomes true of the blood itself. By collecting and estimating the fat absorbed from the intestines through the lacteals—this can be done by means of a cannula inserted into the thoracic duct at the point of its connection with the subclavian and jugular veins—it can be shown that some 60 per cent of the fat is absorbed through the lacteals.† What happens to the remaining 40 per cent is still a mystery. It is supposed that this 40 per cent is absorbed into the portal circulation together with the hexoses and the amino acids.

The fat which finally appears in the blood is either stored (in adipose tissue, etc.) or burned. The details will be discussed in the chapter devoted to the metabolism of fats (Chap. 17).

Absorption of Lecithin.—Several investigators have shown that lecithin is hydrolyzed by the fat-splitting enzyme (or enzymes)—probably lecithinases—of the digestive tract. It would seem, then, that lecithin—and probably phospholipids in general—is not absorbed as such, but is first hydrolyzed into its components, in much the same manner as fat; and, again like fat, is possibly resynthesized immediately after absorption.

Absorption of Cholesterol.—Cholesterol is absorbed in small amounts depending upon the amount of fat (the kind of fatty acids?)

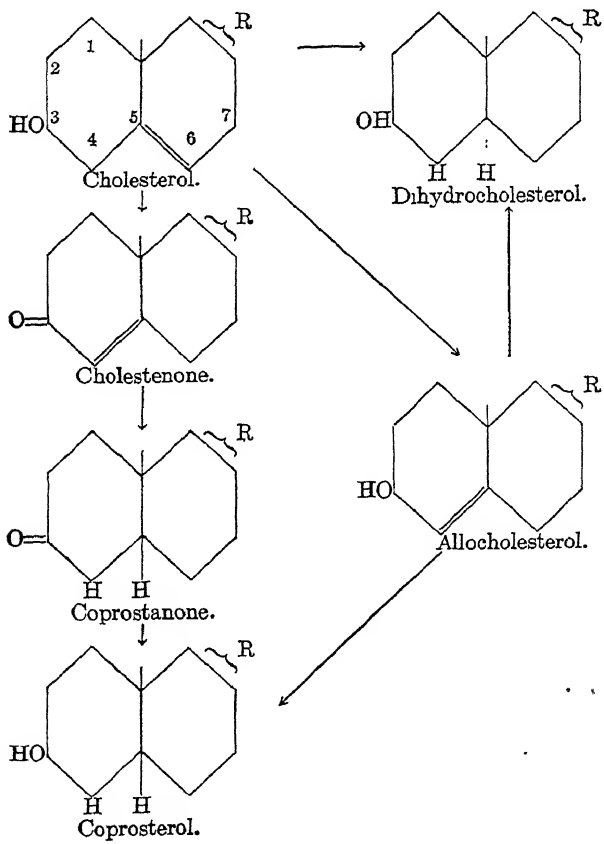
* This concept of fatty acid-bile complex has not gone without challenge.

† More recent work indicates that this figure is too high.

absorbed at the same time. A factor in the absorption may be the solubility of the cholesterol in bile.

Cholesterol is absorbed through the lacteals. The fact that cholesteryl esters have been detected in the chyle suggests that there is esterification of the sterol during absorption. In the blood we find cholesterol as well as cholesterol esters (cholesterol plus fatty acids); and this has led Bloor to suggest that cholesterol may act as a transporter of fatty acids.

It has always been assumed that the coprosterol found in feces is a direct reduction product of cholesterol. However, it is now known that dihydrocholesterol, obtained from cholesterol by reduction, is an isomer of coprosterol. The work of Schoenheimer makes it seem highly probable that the connecting links between these compounds are less direct:



Rosenheim and Webster have presented evidence to show that a dietetic factor, present in brain and other organs, but not in muscle and milk, is essential for the conversion of cholesterol into coprosterol.

To show the delicate responses of the tissues, we know that whereas cholesterol is absorbed by the animal, sitosterol, its isomer in the plant

kingdom, is not. Ergosterol (p. 41) is also not absorbed, although it is absorbed when irradiated.

Absorption of Proteins.—The proteins are absorbed as amino acids; these, like the hexoses, pass directly into the portal circulation. Folin and Van Slyke have shown that the blood always contains amino acids, and that after a meal rich in protein there is a definite increase in the amino acid content of the blood. Abel, using his "vivi-diffusion" technic, arrived at the same result. Here the blood from the portal vein of a dog was passed through collodion tubes immersed in Ringer's solution, and then the blood was returned to the body What diffused out through the collodion tubes contained, among other things, amino acids some of which were actually isolated.

This evidence that the absorption of protein takes place in the form of amino acids is further strengthened by the fact that the injection of protein directly into the blood gives rise to antibodies which can be detected; but no such antibody formation results from the oral ingestion of protein. In fact, there is evidence to point to the view that absorption of even traces of protein through the walls of the intestine may give rise to allergic symptoms.

This problem of "sensitivity" needs emphasis. Where chemical methods fail, immunological methods do lead to the view that very small quantities of protein—what would usually be called "traces"—are absorbed as such by various persons, and in these cases, such absorption may not be unrelated to sensitivity to special protein foods (such as egg white).

The amino acids pass into the liver and thence into the general circulation. The further changes which they undergo will be discussed in the chapter on the metabolism of protein (Chap. 18).

Absorption of Salts and Water.—Active absorption of salts and water takes place in the intestine. Unlike the three principal foodstuffs, no preliminary treatment is needed here before absorption takes place. Both water and salts are absorbed by the blood capillaries.

References

The mechanism of absorption, concerning which much has been written, and about which there are many points of view, is reviewed by Verzar and McDougall in their book *Absorption* (1937); and by Magee, *Physiol. Rev.*, **10**, 473 (1930).

For details of methods employed in the study of the absorptive process, individual papers may be referred to. For carbohydrates, see Cori, *J. Biol. Chem.*, **66**, 691 (1925); Beck, *Ibid.*, **143**, 403 (1942).

The absorption of fat is discussed in much detail by Bloor, *Biochemistry of the Fatty Acids* (1943), p. 85. See, also, Burr and Barnes, *Ann. Rev. Biochem.*, **12**, 157 (1943); Little and Robinson, *Am. J. Physiol.*, **134**, 773 (1941); Barnes, Miller and Burr, *J. Biol. Chem.*, **140**, 773 (1941); Deuel, Hallman and Reifman, *J. Nutrition*, **21**, 373 (1941).

Schoenheimer's work in the formation of coprosterol is described in *J. Biol. Chem.*, **125**, 23 (1938). See, also, Rosenheim and Webster, *Biochem. J.*, **35**, 920 (1941).

For proteins, see Folin and Denis, *J. Biol. Chem.*, **12**, 253 (1912); Van Slyke and Meyer, *J. Biol. Chem.*, **16**, 213 (1913); Luck and Selh, *Biochem. J.*, **19**, 366 (1925); and Bolton and Wright, *J. Physiol.*, **89**, 269 (1937).

CHAPTER 13

BLOOD

THE products of digestion are carried by the blood to the various tissues of the body. The blood also carries the waste products away from the tissues. The hemoglobin of the blood carries oxygen to the

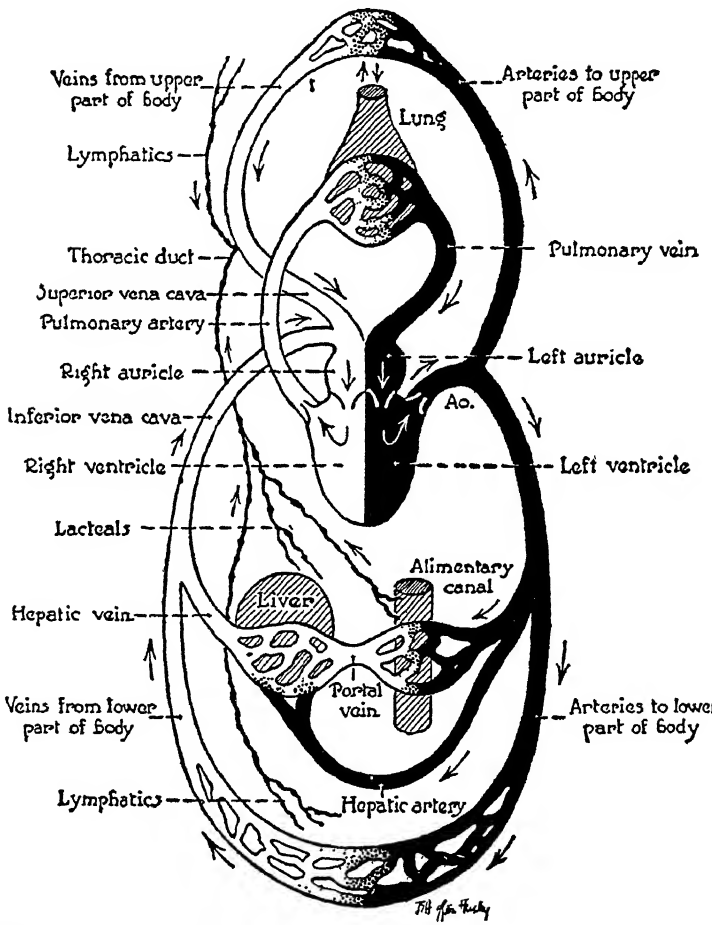


Fig. 65.—Diagram of the circulation of the blood. The arterial, or oxygenated, blood is shown in black, the venous blood, in white. The lymphatics are black knotty lines. (Pettibone, *Physiological Chemistry*, C. V. Mosby Co.)

cells and is involved in the elimination of carbon dioxide from the cells (see Chap. 15). Hormones, the chemical regulators of the body, are also carried by the blood (see Chap. 24). Transportation within

the body, then, is one of the outstanding functions of the blood. (For diagram of the circulation of the blood, see Fig. 65.)

The blood consists of a liquid (plasma) in which are suspended solid components. The suspended materials include the red corpuscles (erythrocytes), the white corpuscles (leukocytes)—of which there are several kinds—and the blood platelets (thrombocytes).* The liquid portion, the plasma, is practically colorless, the red color of blood being due to the red corpuscles suspended in it. By centrifuging blood, the corpuscles can be made to separate; and then it can be observed that they occupy some 40 to 45 per cent of the total volume.

When the blood is allowed to clot, the clear liquid which separates is the serum. Roughly speaking, serum is plasma from which fibrinogen has been removed. If blood is whipped as it is freshly drawn, the fibrin clings to the rod, and a product is obtained which does not clot and which is called "defibrinated blood"; and this is, essentially, blood serum together with corpuscles. This "defibrinated blood" serves quite well for many of the experiments on blood carried out in the laboratory.

Function.—We have already referred to the blood as a transporting medium—for food material, waste, gases, hormones. Blood has a number of other functions of importance. It helps to maintain a delicate osmotic pressure relationship between blood and tissues; it plays its part in the acid-base equilibrium within the body (Chap. 15); it aids in regulating the temperature; and, through its white cells and chemical defense mechanisms, it is of importance in immunological reactions (Chap. 14).

Composition.—The many substances in the blood, with their approximate quantities, are summarized in Table 37. Hemoglobin, fibrinogen, albumin, and globulin are among the chief proteins. Fatty acids (fat), phospholipids, and cholesterol (free and combined) represent the lipids. The sugar that is normally present is glucose. The non-protein-nitrogen (NPN) constituents—substances derived from proteins—include urea, creatinine, creatine, ammonia, and amino acid nitrogen. The inorganic material includes chloride, bicarbonate, phosphate, sulfate, combined in various ways with sodium, potassium, calcium, and magnesium. There may be present small quantities of still other substances, such as the acetone bodies, bile pigments, lactic acid, phenol, iodine, etc. All of these substances are kept in some sort of solution by water, which constitutes about 90 per cent by weight of the blood. The *p*H of the blood is in the neighborhood of 7.4 and its specific gravity is about 1.06.

Clinically changes in the composition of the blood are of great importance. Being the purveyor of materials to and from the cells, a marked deviation from the normal composition of blood may indicate (*a*) a subnormal or abnormal supply of foodstuff from the outside; (*b*) the presence of toxic substances; (*c*) one or more organs which are diseased; etc.

* On the average there are some 5 liters of blood in the individual. In each cubic millimeter of blood there are 5,000,000 red cells, 10,000 white cells and 300,000 platelets.

TABLE 37.—COMPOSITION OF HUMAN BLOOD. (Hawk and Bergeim, *Practical Physiological Chemistry*, P. Blakiston's Son and Co., Inc., Publishers.) (Modified.)

Constituent.	Normal range mg per 100 cc.	Pathological conditions in which increases (unless otherwise noted) may be encountered
Total solids per cent	19–23	Anhydremia. Low in hydremic plethora and anemia
Total proteins (serum) per cent	6 5–8.2	See above Low in nephritis with edema (nephrosis)
Albumin (serum) per cent	4 6–6 7	Low in nephrosis
Globulin (serum) per cent	1.2–2 3	Nephrosis, anaphylactic conditions, malignancy, infections, muscular activity
Fibrinogen (plasma) per cent	0.3–0.6	Pneumonia, infections Low in cirrhosis of liver, chloroform or phosphorus poisoning, typhoid fever.
Hemoglobin per cent (Haden)	15.6	Polycythemia Low in primary and secondary anemia, chlorosis
Iron, as Fe	52	See Hemoglobin
Copper	0 05–0 25	
Total nitrogen per cent	3.0–3.7	Varies chiefly with proteins (albumin, globulin, hemoglobin)
Nonprotein N	25–35	Nephritis, eclampsia, etc See Urea N.
Urea N	10–15	Chronic and acute nephritis, metallic poisoning, cardiac failure, intestinal or prostatic obstruction, some infectious diseases. Relatively low in nephrosis
Uric acid	2–3.5	Nephritis, gout, arthritis, eclampsia.
Creatinine	1–2	Nephritis.
Creatine	3–7	Terminal nephritis.
Amino acid N	3.4–5	Leukemia, acute yellow atrophy of the liver, severe nephritis
Ammonia N	0.1–0.2	Terminal interstitial nephritis
Undetermined N	4–18	Eclampsia.
Glucose	70–100	Diabetes, pregnancy, severe nephritis.
Total fatty acids	290–420	Diabetes, nephritis.
Cholesterol	140–230	Diabetes, nephritis, nephrosis, biliary obstruction, pregnancy. Low in pernicious anemia.
Lipid phosphorus (lecithin)	12–14	Diabetes, nephritis, pregnancy In anemia, low in plasma, high in cells
Total acetone bodies (as acetone)	0.8–5.0	Diabetes.
Acetone + acetoacetic acid (as acetone)	0 3–2 0	Diabetes.
β-Hydroxybutyric acid (as acetone)	0.5–3.0	Diabetes.
Bilirubin	0.1–0.25	Biliary obstruction, hemolytic anemias. Low in secondary anemia.
CO_2 capacity (plasma) vol. per cent	55–75	Respiratory diseases, tetany. Low in diabetes, nephritis.
CO_2 content (arterial blood) vol per cent	45–55	Respiratory diseases, tetany. Low in diabetes, nephritis.
CO_2 content (venous blood) vol per cent	50–60	Respiratory diseases, tetany. Low in diabetes, nephritis
O_2 capacity vol. per cent	16–24	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia
O_2 content (arterial blood) vol per cent	15–23	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia
O_2 content (venous blood) vol per cent	10–18	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
Ascorbic acid	0.8–2 4	Low in scurvy
Lactic acid	5–20	Exercise, eclampsia.
Phenols (free)	1–2	Intestinal obstruction, pernicious anemia, nephritis
Chlorides as NaCl	450–500	Nephritis, cardiac conditions, prostatic obstruction, eclampsia, anemia Low in diabetes, fever and pneumonia.
Sulfates, inorganic as S (serum)	0 9–1.1	Nephritis.
Phosphorus, inorganic as P (plasma)	3–4	Nephritis, Low in rickets. Normal values 1–2 mg. higher in children.
Calcium (serum)	9–11.5	Low in infantile tetany, severe nephritis, parathyroidectomy.
Magnesium (serum)	1–3	No changes noted in disease.
Sodium (serum)	330	Low in cases of alkali deficit.
Potassium (serum)	16–22	Pneumonia, acute infections, occasionally in uremia.
Iodine, γ per 100 cc	8–15	Hyperthyroidism. Low in cretinism

Erythrocytes.—These are the red blood cells (Fig. 66). In appearance, they are biconcave circular disks devoid of a nucleus. Normally, there are some 5,000,000 per cubic millimeter. The erythrocyte is made up of protoplasmic material (stroma) which encloses the pigment hemoglobin. The pigment accounts for more than three quarters of the total solids.

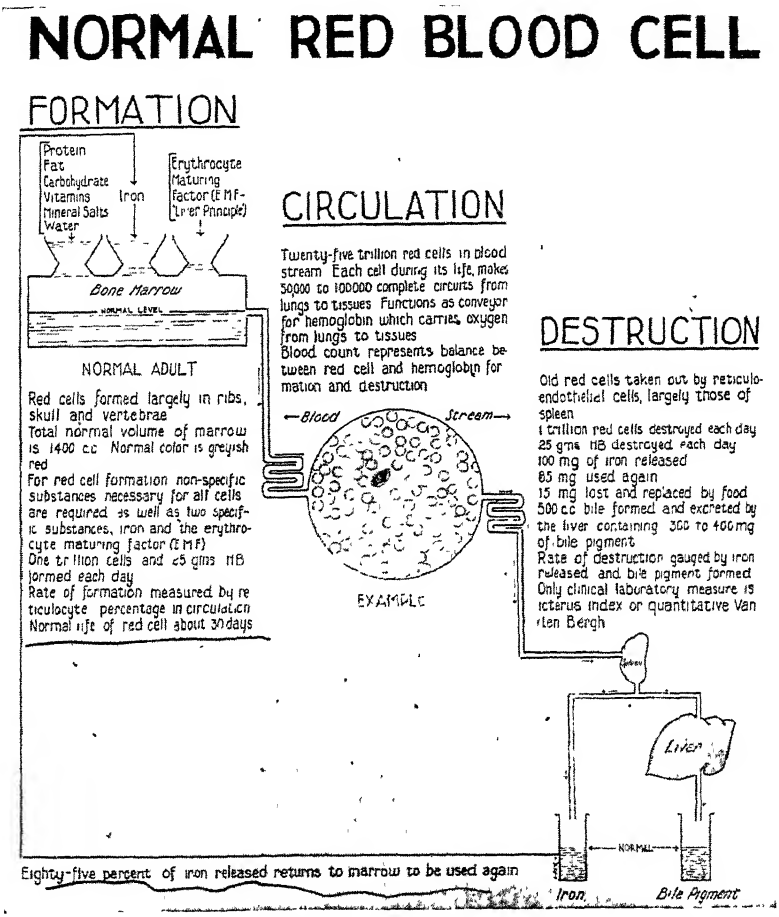


Fig. 66.—The physiology of the normal red cells. A cell lives about forty days, so about one trillion cells and 25 gm. of hemoglobin are destroyed and formed every day. (Haden, *Bull. N. Y. Acad. Med.*, May, 1939, p. 291.)

In various types of anemias there is a notable decrease in the red blood count; and under certain conditions—in fevers, at high altitudes, after severe muscular exercise—there may be a marked increase in the number of red blood cells (polycythemia).

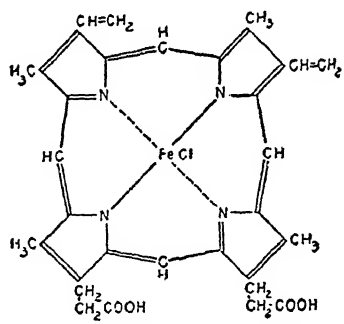
Under certain conditions, the red blood cells undergo destruction, whereby the hemoglobin passes out into the surrounding medium. This process is known as *hemolysis* or "laking of the blood." A simple experimental procedure accomplishes such a result. It is to add water

(or a solution less concentrated in crystalloids than blood—a *hypotonic* solution) to blood. Under these conditions, water will pass into the cells By using a solution more concentrated in crystalloids than blood—a *hypertonic* solution—water will pass out of the erythrocytes and the cells will contract. By using a solution of sodium chloride containing 0.9 per cent of the salt, no contraction or expansion of the erythrocytes occurs. Such a solution is spoken of as *isotonic;* and the osmotic pressure of this solution is equal to the osmotic pressure within the cell.

Hemoglobin.—The physiological rôle of hemoglobin involves its oxygen-carrying capacity and its property as a buffer. These properties will be discussed later (Chap. 15). At this stage we are more concerned with its chemistry.*

Hemoglobin is made up of a protein, globin (which is a histone), and an iron-containing compound known as *heme* (Anson and Mirsky). This heme is also called *hematin.* The chloride of heme, *hemin,* has been known for many years. It can be easily obtained in crystalline form by evaporating a small quantity of blood and a little acetic acid on a microscope slide (a trace of salt is also added) and examining the crystals under the microscope. The characteristic brownish rods so obtained serve as an excellent chemical test for blood.

Hans Fischer has succeeded in synthesizing hemin. Its essential structure involves four pyrrole groups (compare the structure with that of chlorophyll, p. 208).

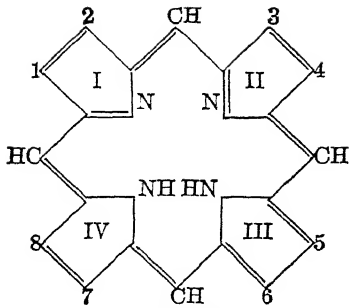


Hemin

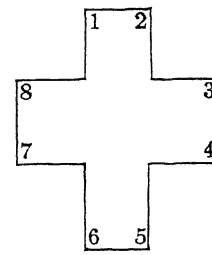
The hemin, then, may be considered as a chloride compound of heme. Both are iron-porphyrin combinations. Porphyrins are substituted porphins and iron-free compounds. The porphin is the "mother substance" containing four pyrrole nuclei.

* Hemoglobins are universally distributed throughout the animal kingdom. Hemocyanins, copper-containing pigments (p. 299), occur only in invertebrates.

Keilin and Wang (*Nature,* **155**, 227 (1945)) point out that hemoglobin is present in the root nodules of leguminous plants. In animals outside of vertebrates hemoglobin has an irregular distribution. "The limiting factor in the distribution of hemoglobin in nature," they write, "is the ability of cells to synthesize the highly specific proteins which, when combined with heme, impart to it the remarkable property of reversable oxygenation."



Porphin



(Porphin in a chemical shorthand devised by Fischer.)

"Heme" not only combines with native (not denatured) globin to form hemoglobin, but it has the property of combining with many nitrogenous compounds to form *hemochromogens*. For example, heme combines with proteins other than globin, with pyridine, with ammonia, etc. Hemoglobin, in this sense, is merely one of a number of possible hemochromogens. These hemochromogens have, as a rule, characteristic absorption spectra, which makes their identification relatively simple. We shall presently see that cytochrome, Warburg's respiratory ferment, peroxidase (Chap. 19), etc., are all examples of hemochromogens.

Hemoglobin combines readily with oxygen to form oxyhemoglobin, and the gas is removed when the pressure is decreased.

$$HbO_2 \rightleftarrows Hb + O_2$$

This property of hemoglobin is of fundamental importance in respiration (Chap. 15). Approximately, 1 gm. of hemoglobin will combine with 1.36 cc. of oxygen. If hemoglobin be oxidized with an oxidizing agent (such as potassium ferricyanide), a product is formed which contains the same amount of oxygen as oxyhemoglobin, but which, unlike the latter, no longer loses its oxygen when the pressure is reduced. This product is *methemoglobin*.

Hemoglobin also combines with carbon monoxide to form a much more stable compound than with oxygen. It combines with carbon dioxide to form, it is believed, a carbamino compound, HbNHCOOH, wherein the union is between the NH_2 of the hemoglobin and the CO_2, and also with nitric and nitrous oxides.

The state of the iron in these compounds, ferric (Fe^{+++}) or ferrous (Fe^{++}), is often determined by behavior toward oxidizing and reducing agents, such as potassium ferricyanide or sodium hydrosulfite.

Using the nomenclature of Peters and Van Slyke, we shall call hemin from which the iron has been removed the "porphyryl" group; and we can then summarize the various hemoglobin combinations, with their approximate structures, as follows:

Porphyryl group	Por
Hemin (formed by action of acetic acid and NaCl on oxyhemoglobin)	Por:Fe^{+++}—Cl
Heme (sometimes called "hematin") (formed by action of NaOH on hemin)	Por:Fe^{+++}—OH
Reduced heme (sometimes called "reduced hematin") (formed by reduction of heme with $Na_2S_2O_4$)	Por:Fe^{++}
Reduced hemoglobin (formed by lowering oxygen tension or by reducing methemoglobin with $Na_2S_2O_4$)	(Globin) (Por:Fe^{++})
Oxyhemoglobin (formed by the combination of oxygen with hemoglobin)	(Globin) (Por·Fe^{++})O_2
Carboxyhemoglobin (the combination of CO and hemoglobin)	(Globin) (Por Fe^{++})CO
Methemoglobin (by the action of ferricyanide on hemoglobin)	(Globin) (Por·Fe^{+++}—OH)
Cyanhemoglobin (action of KCN on hemoglobin)	(Globin) (Por.Fe^{+++}—CN)

The CO_2, as we have seen, probably combines with the NH_2 of the globin portion:

$$HbNH_2 + CO_2 \rightarrow HbNHCOO^- + H^+$$

Just how the globin is attached to the heme portion of the molecule is not known.

The iron is present in hemoglobin to the extent of 0.33 to 0.34 per cent. Assuming 1 atom of iron in the molecule, the smallest molecular weight would be in the neighborhood of 16,000. Svedberg with his ultracentrifuge has determined the molecular weight to be 66,800; which might mean that we are dealing here with four atoms of iron per molecule.

The various interrelationships of these compounds and their products are given in Table 38.

The Anemias.—There are many forms of anemia, and certainly no attempt will be made to treat the subject clinically. But in the various forms which are known, we deal with a reduction in the number of red blood cells or a reduction in the amount of hemoglobin. Such a loss immediately affects the amount of oxygen which can combine with hemoglobin. The anemias, according to Krumbhaar, are due (1) to disorder of erythrocyte formation; (2) to loss of blood; and (3) to excessive destruction. Both nutritional anemias and pernicious anemia would come under (1). In anemias associated with formation of giant red blood corpuscles (macrocytosis), characteristic of pernicious anemia, the liver extract treatment of Minot and Murphy has proved of great benefit.* Where the deficiencies are associated with "an acquired microcytosis, especially if hypochromic," the treatment indicated is one of iron (in the form of one of its many compounds).

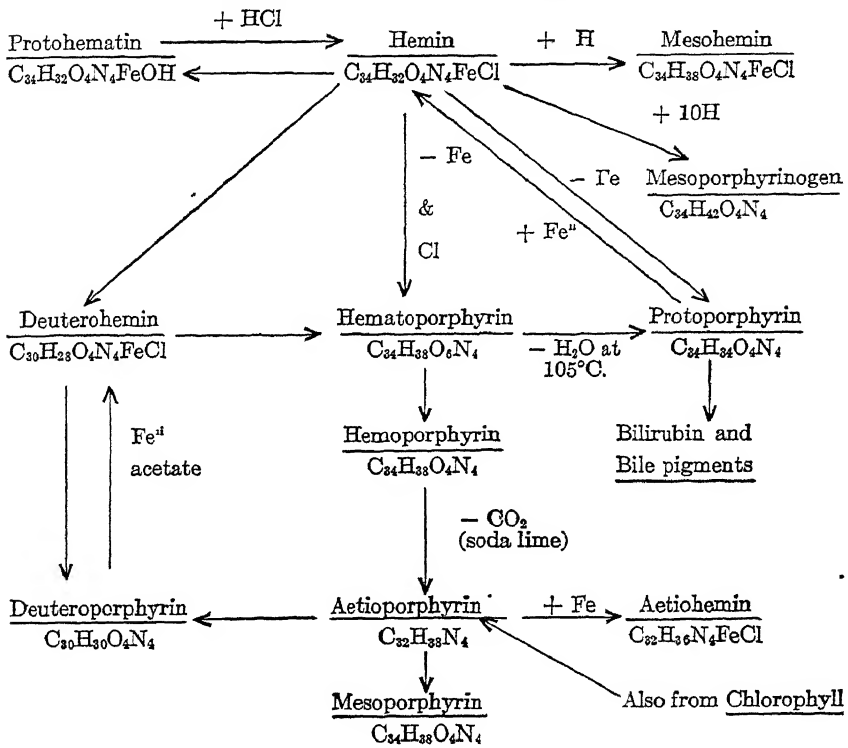
Nutritional anemia will be discussed in another chapter (Chap. 21). But the dramatic recoveries in pernicious anemia, resulting from the pioneer researches of Whipple, Minot, Murphy, Castle, etc., make further comment pertinent at this point.

Whipple, as a result of many years of work on blood regeneration, came to the conclusion that pernicious anemia results from improper formation of the stroma of the red corpuscles. In this disease the primitive blood cells crowd the bone marrow. Minot and Murphy showed that feeding large amounts of liver, kidney or certain fractions

* See also folic acid, footnote, p. 172.

obtained from liver, produced a prompt increase of young blood cells, particular reticulocytes, in the peripheral blood. The active principle in liver promotes the growth of the primitive cells. The increase in reticulocytes continues for about nine days; then there is a decrease. In the meantime, the red blood cells begin to increase. The two are in inverse ratio. Beyond 3,000,000 red blood cells per cubic millimeter, the reticulocyte response is slight (Fig. 67).

TABLE 38—HEMIN-PORPHYRIN RELATIONS.



A striking symptom in pernicious anemia is the almost invariable absence of free hydrochloric acid in the gastric juice of the patient. Castle postulated that "the significant defect in the patient with pernicious anemia is an inability to carry out some essential step in the process of gastric digestion. . . . " The daily administration of 200 gm. of beef muscle as such, or after digestion with pepsin-hydrochloric acid, to patients suffering from pernicious anemia led to no improvement. The daily administration of 300 cc. of human gastric juice (secreted by normal fasting subjects in response to histamine injection) proved no better. However, when normal human gastric juice and beef muscle were given together "reticulocyte responses, increases of the red cells and hemoglobin, and clinical improvement promptly appeared."

The improvement, according to Castle, depends upon two factors: one factor is in beef muscle (extrinsic), and the other factor is in nor-

mal gastric juice (intrinsic). These two "factors" react to form a "something" which is absorbed, stored in the liver and has this stimulating action on blood formation. Castle's belief that the "extrinsic" factor is one of the vitamins connected with the heat-stable B_2 complex remains to be confirmed.*

The Chemistry of the Substance Involved in Pernicious Anemia.—The pioneer work in this field was carried out by Cohn and his associates. The active principle is soluble in water, insoluble in ether, and

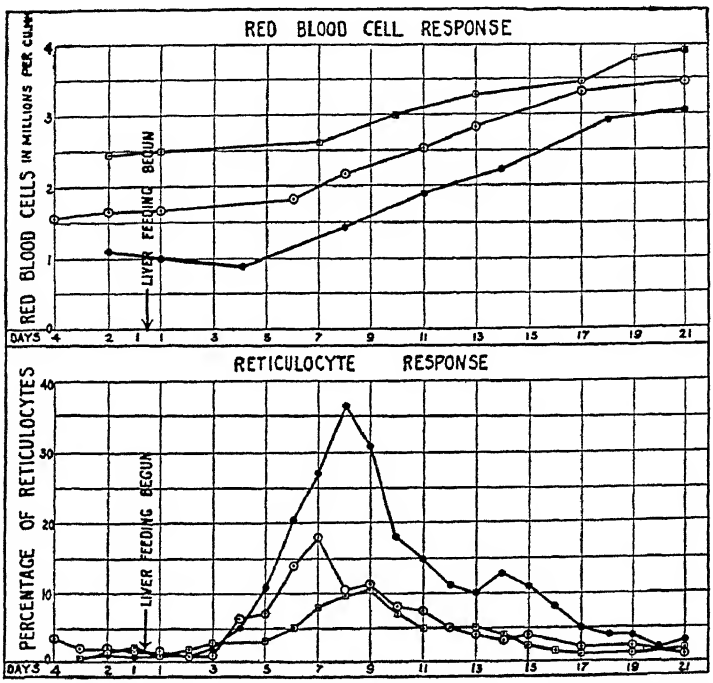


Fig. 67.—The effect on the reticulocytes in pernicious anemia of feeding daily 220 gm of liver pulp to each of three patients with different red blood cell levels. In general, the highest blood cell count is accompanied by the lowest reticulocyte count and vice versa. (Minot, Murphy, and Stetson, *Harvey Lectures*, Ser. 23, Williams and Wilkins Co., Publishers.)

precipitated with alcohol. It contains neither iron, protein, carbohydrate nor lipid. Further chemical work was done by Dakin and West who believe the hematopoietic substance to be a peptide or an

* Beef muscle (representing extrinsic factor) and normal human gastric juice (intrinsic factor) administered to patients with pernicious anemia gives a beneficial response within ten days. In the place of beef muscle, milk, eggs, liver, yeast, rice polishings and wheat germ, all sources of the vitamin B complex, can be substituted. This hematopoietic reaction—this building of blood from food and body products—does not occur if in the place of any of the extrinsic factors one or more of the known vitamins of the B complex are used. Castle, however, is still of the opinion that the extrinsic factor is a member of the vitamin B complex, but it has yet to be identified (Castle, Ross, Davidson, Burchenal, Fox and Ham, *Science*, **100**, 81 (1944).)

There is some evidence that the metabolism of tyrosine is abnormal in cases of pernicious anemia. In support of this theory, it has been shown that there is an increased elimination of keto acids and hydroxyphenyl compounds in this disease.

association product of a peptide. Subbarow is of the opinion that the pyridine ring is important. Erdos claims to have shown that the active substance is an amino acid complex with three free COOH groups and contains sulfur and phosphorus. Its molecular weight is said to be 6,000.

White Cells or leukocytes, of which there are several varieties, are, as a rule, larger in size than erythrocytes, and, unlike the latter, possess a nucleus. They also possess the power of ameboid movement, whereby they can leave the cells and wander into surrounding tissues. They may number 10,000 per cubic millimeter, the proportion of leukocyte to erythrocyte being roughly 1:500.

These leukocytes, being typical cells, are composed of characteristic cellular material—protein, lipid, etc. They act as phagocytes, thereby defending the organism against invading bacteria.

Blood platelets or thrombocytes are believed to be of importance in blood coagulation. They are round oval disks, in diameter about one third that of the erythrocytes, and they may number some 300,000 to the cubic millimeter.

Blood Plasma.—The plasma is the blood from which the corpuscles have been removed. It, therefore, is devoid of hemoglobin, for example, but otherwise contains practically everything found in whole blood. Of the 9 per cent of solids which are present, some 7 per cent is due to proteins. The proteins, in approximate relative percentages, are albumin, 58; α-globulin, 14; β-globulin, 13; γ-globulin, 11; fibrinogen, 4.

Plasma Proteins.—Aside from fibrinogen, which plays a specific rôle in blood coagulation, the proteins of the blood (and this applies more particularly to the albumin and the globulin fractions) maintain the water balance between the blood and tissues. This property is dependent upon the attraction for water which these proteins have. While it is quite true that the osmotic pressure of the plasma proteins is almost negligible when compared with the crystalloids present, nevertheless the latter, unlike the proteins, do not play a rôle in the distribution of water, due to the fact that they pass freely through the cellular walls.

That these proteins—and more particularly the albumin, because it has a smaller molecule and is present in larger quantity*—are important in the distribution of water is evident from the fact that patients with a deficient amount of serum albumin suffer from edema (Fig. 68). An experimental procedure of producing edema points to a similar conclusion. The procedure is known as "plasmapheresis." This consists in removing blood from the animal and reinjecting the washed corpuscles bathed in Ringer-Locke solution (a solution of inorganic salts comparable in osmotic pressure to that of the blood itself). The amount of plasma proteins removed in this way will depend upon the amount of blood removed. Leiter found by this method that when the plasma proteins reached a level of less than 3 per cent, edema developed.

* About 60 per cent of plasma protein is composed of albumin, "but it is responsible for nearly 80 per cent of the blood's osmotic efficiency" (Cohn).

The edema common in nephrosis has been associated with loss of plasma protein, and with a correspondingly lowered osmotic pressure.

Weech has pointed out that edema rarely appears before the albumin is below 2 per cent. Between globulin and edema there is little, if any, correlation (see Fig. 68). Weech defines his serum albumin as that fraction of the protein of serum which remains in solution after half saturation with ammonium sulfate; the fraction which is precipitated is the globulin Chemically, these are not very sharp separations.

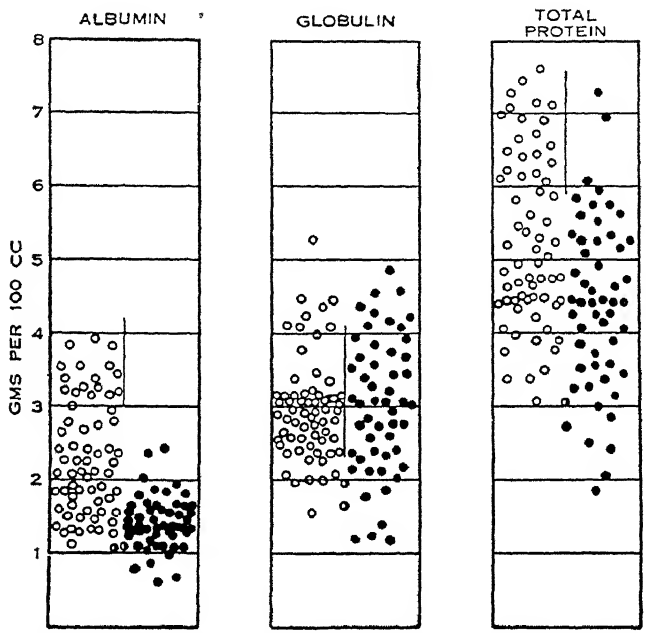


Fig. 68.—The relation between plasma protein concentration and edema in dogs. Open circles indicate estimations when no edema was present; black circles refer to determinations when edema was present; vertical lines in the middle of each column indicate the range of normal variation. (Weech, *Bull. N. Y. Acad. Med.*, Feb., 1939, p. 63)

Of secondary importance is the buffering power of the albumin and globulin. Van Slyke and his co-workers have shown that, among the proteins of the blood, the hemoglobin is the important buffering agent. Nevertheless, the albumin and globulin do help to some extent. The isoelectric point of albumin is given as *p*H 4.8, and that of globulin, *p*H 5.5; and since the blood itself is at a *p*H of 7.4, these proteins are in combination with bases.

When we come to the problem of the individuality of these various proteins in blood plasma, we get into difficulties. The fibrinogen is apparently a specific protein, in the sense that it alone undergoes the change to fibrin during the course of blood coagulation. In its general properties, fibrinogen is often classified as a globulin; but, though insoluble in water in the absence of salts, it coagulates at a lower temperature than do globulins in general (it coagulates at 55° C.), and

it is more easily precipitated by the addition of salts. For example, half saturating the solution with sodium chloride will precipitate the fibrinogen; but more sodium chloride is needed for the precipitation of the usual type of globulin

The serum albumin is soluble in water and, unlike the globulin fractions, requires a solution fully saturated with ammonium sulfate before it is precipitated; and, again unlike the globulin fractions, it is not precipitated either by dilution or by dialysis of the plasma.

The fraction containing serum globulin consists of several proteins. Such names as "euglobulin" and "pseudo-globulin" have been used. These separations have been accomplished by fractional precipitation with salts, particularly ammonium sulfate.

Using an improved electrophoresis apparatus—an apparatus which involves the use of the electric current to send negative colloid particles toward the positive pole, and vice versa—Tiselius found serum to consist of albumin and three globulins, which he called α, β, and γ. The isoelectric point of the γ variety is *p*H 6.0, whereas the isoelectric points of the other two are 5.1 and 5.6. While all three have approximately the same molecular weight, their mobilities in alkaline solutions are quite different.

Origin of Plasma Proteins.—These proteins originate in the protein (amino acids) of the food, and their synthesis, to a large extent, occurs in the liver. They are in what Whipple calls "dynamic equilibrium" with the other proteins of the body; that is to say, the proteins of plasma, liver and other tissues are in constant exchange. Feeding rats isotopic amino acids, Schoenheimer confirmed this view: the concentration of isotopic nitrogen in the plasma proteins was slightly lower than that in the liver, but somewhat higher than that of the internal organs. "They demonstrate," he writes, "the continuous chemical interactions of serum proteins with body proteins and diet."*

Protein Deficiency.—Plasma protein in amounts below normal (5.5 gm. protein per 100 cc. of blood, or below) (hypoproteinemia) is also an indication of loss of body protein.

The hypoproteinemia may be the result of one or more of the following: insufficient intake of protein; poor utilization; excessive loss of blood, and, therefore, of plasma proteins.

In nephritis there is a marked loss of blood albumin, giving rise to albuminuria (in the urine).

The hypoproteinemia is often accompanied by edema (p. 263) and anemia. The edema results from an increased interstitial fluid volume, because with less protein within the blood vessels, less liquid is drawn into them than would normally be the case.

If the blood volume is sufficiently reduced, what is known as "shock" develops.

Shock, as observed clinically (and on the battlefield), is due in part to a decrease in the volume of the blood, the result of loss of blood and, more particularly, blood proteins. The injection of plasma (containing

* Incidentally, in contrast to the proteins of the plasma and organs, hemoglobin has a low concentration of isotope.

the proteins), or the injection of plasma proteins themselves may increase the plasma volume once more, because these proteins draw water from the tissues into the blood. Here the albumin of the plasma plays the major rôle.

Clinical Use of Blood Plasma.—During the war period blood plasma has been used in enormous quantities. Following shock, hemorrhage or burns, blood transfusion has been a common practice. Blood plasma, unlike whole blood, requires no special "typing," and hence a pooled supply (the "blood bank") can be used more or less indiscriminately. In shock and in burns the plasma transfusion is very effective. It may be less so in the case of a hemorrhage with a considerable loss of red cells; then whole blood (of the right kind) may be the more desirable.

TABLE 39.—FRACTIONATION OF BLOOD. (Cohn, *American Scientist*, **33**, 61)

	Principal function.	Principal protein related to function	Concentrated in plasma fraction	Established clinical use.
RED CELLS (45% of blood containing 30% hemoglobin)	Respiratory	Hemoglobin		Whole blood or red cell transfusion.
PLASMA (55% of blood containing 7% proteins)	Osmotic regulation of blood volume	Albumin	V	Shock, burns, edema, hypoproteinemia.
	Blood coagulation	Fibrinogen	I	Fibrin films as dural substitutes.*
		Prothrombin, thrombin	III-2	Fibrin foam and thrombin as hemostatic agent
	Immunological	Blood grouping globulins	III-1	Blood grouping.
		Complement $\frac{C'1}{C'2}$	III-2 IV	
		γ-globulins	II	Measles prophylaxis.
	Carbohydrate and lipid solution and transport	β-globulins	III	
		α-globulins	IV IV	
	Regulatory	Fibrinolytic enzyme	I, III-2	
		Phosphatase and other enzymes	III, IV	
		Hypertensinogen	IV-3, 4	
		Thyrotropic hormone	IV-3, 4	
		Gonadotropic hormones	III, VI	

* Substitutes for outermost membrane of brain.

The blood bank is prepared, suitably preserved and used when needed.*

* Examples of methods used in the preparation of suitable blood plasma products are the following:

The blood cells are separated from the plasma by sedimentation or centrifugation. For every 500 ml. of blood 50 ml. of a 5 per cent solution of sodium citrate is added. As a bactericide, merthiolate (a thiosalicylate containing mercury in organic combination), 1 to 10,000, is added. A 50 per cent dilution of the plasma with saline is used in order to keep the blood from becoming too viscous and to prevent the possible precipitation of the fibrinogen. The pooled plasma is preserved at 3–5° C.

Dried blood plasma has also come into much use. The fluid plasma is sprayed into a distilling flask maintained at 15 mm. of mercury and at 45° C. glucose is added as a dispersion medium and for increasing the solubility of the product.

In one method, 40 gm. of plasma residue and 8 gm. of glucose are dissolved

Fractionation of Plasma.—Largely through the work of Cohn and his associates, the proteins in plasma have, in a sense, been separated, or, better, grouped into "fractions," each "fraction" representing not necessarily a homogeneous substance, but one which is reproducible and can be used clinically.

The process as developed separates the plasma into five fractions. Table 39 supplies details.

Blood Coagulation.—This process is still very far from being completely understood. We shall first give a summary of what is believed to be the series of reactions involved.

$$\text{Prothrombin} \xrightarrow[\text{Calcium salts}]{\text{Thromboplastin}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$

Or, in some more detail (Fig. 69).

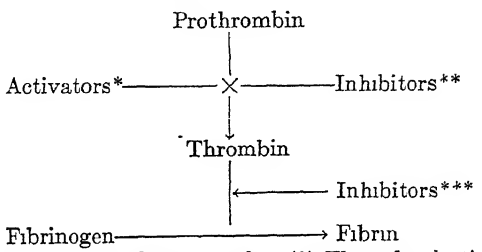


Fig. 69.—* Activators: (1) Calcium salts (2) Thromboplastic agent
** Inhibitors: Antiprothrombins, including heparin and other sulfonic substances.
*** Inhibitors: Antithrombins, including hirudin.
Nonspecific factors: Temperature, *p*H, salt concentration, "wetting," etc. (Ferguson.)

Just what kind of combination occurs between the prothrombin, calcium and the thromboplastic factor (cephalin-protein complex) is not at all clear. Using radioactive isotopic phosphorus (P^{32}_{15}) in the form of Na_2HPO_4, the latter was administered to rats and the radioactive thromboplastin isolated. Prothrombin was converted to thrombin by mixing the former with a calcium chloride solution and a suspension of the radioactive thromboplastin. The radioactivity of the thrombin (obtained by centrifugation) showed that 1 mg. of prothrombin had taken up only 0.19 micrograms (or 0.02 per cent) of radioactive P. Whether this is significant remains to be seen.

When blood escapes from the blood vessels it "clots" or "coagulates." The "clot" consists largely of a protein, fibrin, which is highly insoluble, and which does not exist as such in the intact blood.†

Based on X-ray analysis, Astbury is of the opinion that the change

in 400 ml. of sterile distilled water for each transfusion. This is the equivalent of about 450 ml. of fresh plasma in protein content (a very important factor); 600 mg. of sodium sulfathiazole (p. 284) is added as a preservative.

Other methods dry the plasma by processes involving freezing and drying *in vacuo*.

† A fact not yet explained satisfactorily is that crystalline trypsin can clot

of fibrinogen to fibrin is a change in molecular shape comparable to what happens when muscle contracts.

Fibrinogen.—The precursor of fibrin is fibrinogen, a protein which is in the dissolved state in blood. This fibrinogen may be obtained from plasma by taking calcium-free blood (blood to which 0.1 per cent of sodium oxalate has been added to prevent clotting), centrifuging (to separate the corpuscles), and adding an equal volume of a saturated solution of sodium chloride to the plasma The precipitate so obtained can be purified by redissolving in sodium chloride solution (1 per cent) and reprecipitating with an equal volume of a saturated solution of the salt.

The purified fibrinogen can be redissolved in salt solution (1 per cent), and a typical fibrin clot will be obtained when such a solution is mixed with blood serum or any extract containing "thrombin."

When the liver is excluded from circulation or injured by poison or disease, a notable decrease in fibrinogen becomes evident; the liver, therefore, is considered as the seat where fibrinogen is manufactured.

Prothrombin and Thrombin.—By allowing blood to clot and precipitating the serum proteins with an excess of alcohol, a product is obtained which is dried and extracted with water. This aqueous extract mixed with a solution of fibrinogen causes clotting. The extract contains "thrombin." The latter may also be obtained by extracting fibrin with sodium chloride solution (8 per cent).

If the operation is repeated, but the freshly drawn blood is run directly into alcohol instead of first being allowed to clot, no active thrombin preparation will be obtained. In normal blood, then, we do not have thrombin, but some precursor to which the name "prothrombin" has been given.

As has been shown elsewhere (p. 191), the content of prothrombin is associated with the amount of vitamin K in the body. On a diet deficient in vitamin K chicks develop a hemorrhagic condition. At the same time, the amount of prothrombin in the plasma is reduced. In obstructive jaundice and in experimental liver injury, with the development of the hemorrhagic condition, there is also a parallel decrease in the prothrombin content. In obstructive jaundice, the reduced capacity for fat absorption—and vitamin K is fat-soluble—may perhaps account for the reduction in the amount of prothrombin. In liver injury, the reduction in the quantity of prothrombin may be due to the fact that

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citrated plasma and can activate prothrombin without, apparently, added calcium ion, though the presence of the latter causes the trypsin to be much more active.

The citrated blood is similar to oxalated blood; in the former unionized calcium citrate, and in the latter, precipitated calcium oxalate are formed.

Chargaff and Ziff have shown that ninhydrin (p. 47) when added to fibrinogen solutions of plasma produce typical fibrin clots.

A new theory of blood clotting, based on the coagulating power of trypsin, has been advanced by Ferguson. In blood there is a tryptase comparable to trypsin. Normally an "inhibitor" prevents the tryptase from acting. However, when the "inhibitor" is removed, the tryptase "is the 'missing link' in the thromboplastic action."

Normally, then, the enzyme is not in an active stage; but "damage" introduces new conditions favorable to activation of the enzyme. In this theory the anticoagulants are, in reality, tryptase inhibitors.

the liver is the seat of manufacture of this substance. It is believed that the thrombin and prothrombin, like the fibrin, are proteins, probably containing some carbohydrate. Is it then some enzyme of high specificity which converts fibrinogen into fibrin?*

For various purposes—in jaundice, liver injury, etc.,—the determination of prothrombin becomes important. Several methods are available. One of the simplest is that suggested by Quick. In principle, this method involves determining the time required to clot recalcified plasma in the presence of excess quantities of thromboplastin (see below). The less prothrombin present the longer the coagulation time.

Calcium Salts.—We have already seen that the addition of sodium oxalate to blood prevents it from clotting. The oxalate precipitates the calcium without which clotting cannot take place. If calcium ion is added to a calcium-free blood, clotting takes place.

It is believed that the calcium ion is involved in the conversion of prothrombin to thrombin. Once the thrombin is formed, no calcium ion is needed for the production of a clot; that is to say, when thrombin and fibrinogen are mixed, the clot is formed, even in the presence of oxalate.

Thromboplastin.—At one time or another this substance has been called thrombokinase, thrombozyme, cytozyme, etc. It occurs in most animal tissues, and it is released whenever a wound is produced, thus giving rise to extravascular clotting. It is also present in the blood platelets (in some combined form?); and when released, under certain conditions, intravascular clotting may take place.

According to Chargaff, two types of substances are extracted from tissues depending upon the solvent used. If the material is extracted with water we get the thromboplastic protein; if with a fat solvent, then the thromboplastic lipid (minus the protein) is obtained.

The lipids in the thromboplastic protein are quite complex chemically. They include alcohol-soluble and alcohol-insoluble (cephalin?) phosphatids.

A highly active thromboplastic protein—a lipoprotein?—has been obtained from lungs. It contains, among other things, some ribose nucleic acid.

The thromboplastic lipid, it is true, is associated with cephalin, but since the active material can be removed with alcohol, leaving behind the cephalin, the activity is obviously not due to this phosphatide.

Anticoagulant Factors.—Many influences can retard or inhibit clotting; such anticoagulant factors include physical agents (cold, dilution, excess salts, protein precipitants, etc.); decalcifying agents (oxalate, citrate, fluoride); lipid solvents which remove the cephalin factor; heparin; dicoumarol. These anticoagulants may act either by preventing thrombin formation or by preventing the reaction between thrombin and fibrinogen.†

* Quick is of the opinion that prothrombin consists of two components joined by calcium.

† Still another method of preserving blood and preventing clotting has been suggested by Steinberg, who used a phenol-formaldehyde resin, active as an ion-exchange agent and used in the preparation of ion-free water.

Antithrombin and Antiprothrombin Substances.—As has already been pointed out, the blood clotting mechanism can be divided into two parts (a) the formation of thrombin from prothrombin; and (b) the conversion of fibrinogen into fibrin. Inhibitors or anticoagulants of part (a) are called *antiprothrombic;* and those of part (b) are called *antithrombic.*

Heparin or an Antiprothrombin.—A substance first obtained by Howell from the liver which prevents blood from coagulating, and to which the name "heparin" (Fig. 70) has been given, has no inhibitive action on thrombin itself (as hirudin has), but prevents the conversion of prothrombin to thrombin. Jorpes, among others, has investigated its chemistry and believes it to be a chondroitin-sulfuric acid combina-

Fig. 70.—Crystalline barium salt of pig heparin. Magnification, × 400. Photomicrograph, courtesy of Dr. D. H. Hamly. (Jaques, Waters and Charles, *J. Biol. Chem.*, **144**, 299.†)

tion (see Chap. 22), composed of a uronic acid—of the glucuronic acid type—hexosamine and ester sulfate.

Heparin is the most potent anticoagulant known. Its effects are destroyed (or neutralized) by the protamine salmine.*

The heparin in any one species seems to display a definite chemical individuality; but the evidence points to different heparins in different species.

Heparin has been suggested as a substance which might be used to prevent thrombosis.

Hirudin or an Antithrombin.—Blood-sucking animals (leeches, ticks, etc.) secrete a substance which prevents coagulation. An extract when mixed with thrombin will prevent coagulation with fibrinogen.

* The explanation of the anticoagulant properties of heparin needs some modification, according to the work of H. P. Smith and his associates and of Astrup. If the substances which are used and which are involved in the coagulation process (fibrinogen, prothrombin, calcium ion, and thromboplastin) are pure enough, then heparin has no inhibitory action. Some factor, present in the serum albumin fraction, is necessary for the inhibitory action of heparin.

Dicoumarol (also called *dicoumarin*), the hemorragic substance in sweet clover disease (p. 271), is also an inhibitor of blood coagulation, and interferes with the formation of prothrombin. It may, therefore, also be looked upon as an

This antithrombin, known as "hirudin," is present (or some such substance like it is present) in small quantities in blood; but what its function, if any, is in the process of blood coagulation is not clear.

Synthetic rather than natural anticoagulants are also known. Two of these substances are the sodium salt of cellulose disulfuric acid $(C_6H_8O_{11}S_2Na_2)_x$, and the potassium salt of polyvinyl sulfuric acid $(C_2H_3O_4SK)_x$. The sulfuric acid esters of the cerebrosides (Chap. 3) also show anticoagulant activity.

Variations in the Time of Coagulation.—Normally, of course, blood does not coagulate within the body. However, in certain diseases (arteriosclerosis, varicose veins, etc.) such a coagulation may occur (thrombosis). On the other hand, whereas blood when shed usually coagulates within five minutes or so, in some rare cases the coagulation time is much prolonged, and in a few cases the blood does not clot at all. Here we are dealing with a disease known as *hemophilia*. The disease is "carried" by the female, who is not the sufferer, and "transmitted" to the male, who is the sufferer (see Fig. 71).

The delayed clotting time can often be restored to normal values by repeated transfusions of blood.

The cause of bleeding in hemophilia is not known. There seems to be no lack of prothrombin or fibrinogen. Just how much thromboplastin is available from platelets and other sources when tissue disruption occurs is not clear.

In the laboratory, coagulation may be hastened by bringing the blood in contact with some outer surface—handkerchief, bandage, etc.; or by gentle heat (using hot cloths). On the other hand, coagulation

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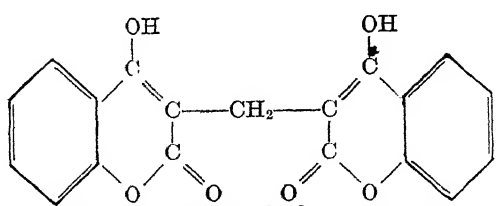
antiprothrombin type of substance. However, it is not a compound like heparin which is a normal constituent of the body.

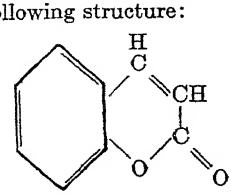
Like heparin, its use has been suggested in the prevention of thromboses.

The formula for dicoumarol may be written in the keto form (as shown on p. 195) or in the enol form:



3,3′-Methylenebis (4-hydroxycoumarin) or Dicoumarol.

Coumarin itself has the following structure:



There is some evidence for the belief that the active metabolic product produced by dicoumarol may be salicylic acid.

The synthesis of dicoumarol is summarized in the appendix (p. 569).

may be retarded or prevented in a number of ways, some of which have already been indicated: cooling, precipitating the calcium ion (with sodium oxalate) or preventing its ionization (with sodium citrate); addition of large quantities of neutral salts; the addition of hirudin, dicoumarol or heparin; the addition of snake venom; etc.

Tests for Blood.—Two of the tests depend upon color production, the result of oxidation: the *guaiac test* and the *benzidine test*. The former involves the use of guaiac dissolved in glacial acetic acid, to which are added the blood and hydrogen peroxide; a blue color is formed. The benzidine test involves the use of an acid (glacial acetic) solution of benzidine mixed with blood and hydrogen peroxide. A blue or green color develops.

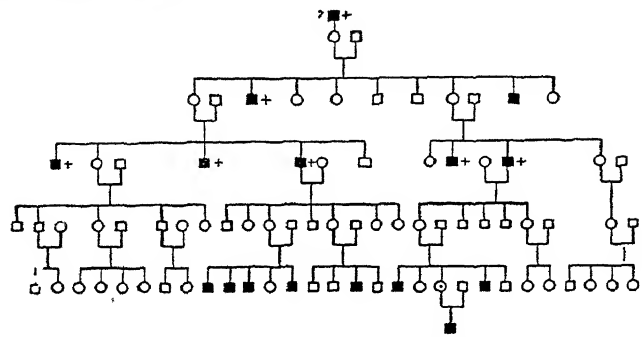


Fig. 71.—The family tree of the Hawkins-Cooper family. This family has lived in southern Illinois since before the Civil War. The squares represent males; the black squares, those with hemophilia; the black squares with crosses, those who bled to death; the circles, females, the circles with inner dots, transmitters of hemophilia. The chart shows fifteen patients known to have hemophilia and one whose condition is questionable. All eight persons in this generation who have hemophilia have been seen. (Birch, *J. Am. Med. Assoc.*, **99**, 1566.)

The best chemical test—a test which definitely indicates the presence of blood, though it does not distinguish human blood from other varieties—is the *hemin test*, to which reference has already been made (p. 258).

The immunological test distinguishes human blood from other varieties. Rabbits are injected with human blood serum, over a period of several days and in increasing quantities. The rabbit develops an antiserum. Blood is withdrawn from the animal and its serum mixed with human serum under examination. A turbidity, gradually changing to a flocculent precipitate, indicates the presence of human blood.*

Interstitial Fluid.—Surrounding the intra-cellular fluid of the cells proper, there is what is called the extracellular fluid (Gamble), which consists of (a) the blood plasma and (b) *the interstitial fluid*. This interstitial fluid includes the *lymph* (Fig. 72).

The lymph, formed probably from the plasma of the blood and filling tissue spaces, acts as a "medium" between the blood and cells. In composition it resembles the plasma (Table 40). Lymph capillaries, abounding in the tissue spaces, carry away the lymph into vessels which become larger, and which unite at the thoracic duct, which in

* See appendix, p. 570

turn, empties into the subclavian vein; so that ultimately the products in the lymph find their way into the general circulation.

One of the important functions of the lymph system is a defense against inflammatory processes.

Blood Analyses.—Table 37 (p. 256) gives the composition of the blood. (See also Table 41.) For details with regard to such analyses, practical texts have to be consulted (see the references at the end of the chapter). However, a few brief remarks at this stage may not be amiss.

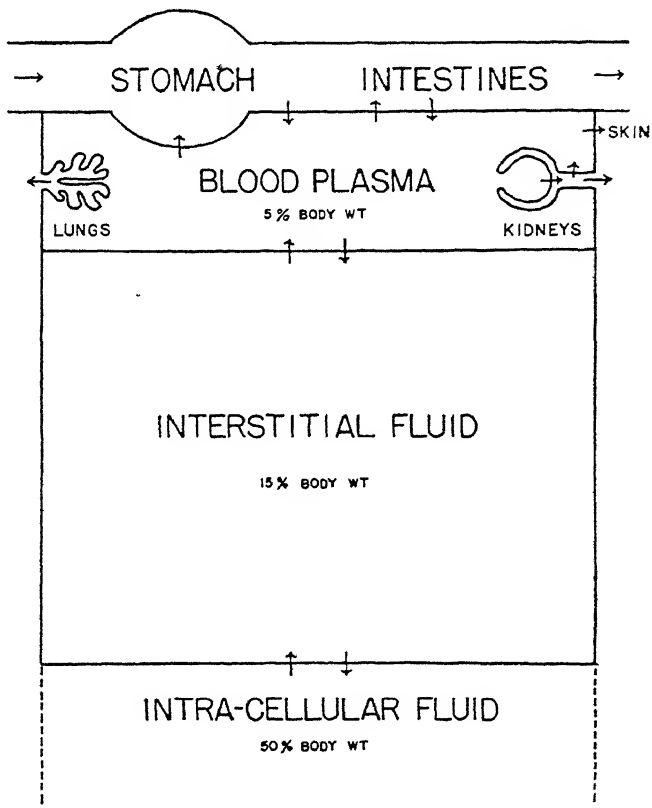


Fig. 72.—*Interstitial Fluid* (Gamble, *Extracellular Fluid*, Harvard Med. School).

The importance of blood analyses as aids in clinical diagnosis has been realized for a long time. To take but a few examples at random: a low iron content in the various anemias (p. 260); a hyperglycemia in diabetes (p. 493); low phosphorus and increased serum phosphatase in rickets (p. 178); decreased plasma prothrombin in obstructive jaundice (p. 192); increased blood NPN (urea N, creatinine, uric acid, etc.) in renal impairment (p. 472); etc.

For a long time, progress in blood chemistry was more or less at a standstill, due to the fact that the methods were not adapted to small quantities. With the introduction of micro methods, more particularly

TABLE 40.—COMPARISON OF THE CONCENTRATIONS OF SOME OF THE CONSTITUENTS IN PERIPHERAL (CERVICAL) LYMPH AND BLOOD PLASMA OF THE DOG UNDER NORMAL CONDITIONS (Heim, *Am J Physiol.*, **103**, 553.)

	Protein (Kjeldahl).	N P N	Urea.	Uric acid.	Creatinine	Sugar	Amino acids.	Chlorides as NaCl.	Phosphorus.		Calcium
									Total.	Inorganic.	
	per cent	mg. per 100 cc	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg per 100 cc.	mg. per 100 cc.	mg per 100 cc.	mg. per 100 cc	mg. per 100 cc
Plasma:											
Average.....	6.18	32 6	21 7	Trace	1 37	123 0	4 90	678	22 0	5 6	11 70
Range.....	(5 54–7.23)	(21.1–46 0)	(17 9–28.0)	.	(1 22–1 54)	(112 0–143.0)	....	(649–721)	(18 3–26 1)	(4 4–6 9)	(10 85–12 95)
Lymph:											
Average.....	3 32	34 8	23.5	Trace	1 40	132 2	4 84	711	11 8	5 9	9 84
Range.....	(1.38–4.57)	(19 8–45.4)	(19.8–33.0)	.	(1 28–1 49)	(107 0–144 0)	.	(690–730)	(10 2–13 7)	(4 7–7 3)	(8 93–10 84)

with the introduction of the colorimeter and the photoelectric colorimeter, blood chemistry became a very important adjunct to clinical diagnosis.

TABLE 41.—NORMAL AND ABNORMAL BLOOD CHEMISTRY FINDINGS (mg. per 100 ml.). [*Merck Manual* (1940).]

	Glucose.	NPN.	Urea N.	Creatinine	Uric acid.	CO_2 comb. power.	Cholesterol.	Sodium chloride.
Normal	70–100	25–25	10–15	1–2	1–3	55–75	150–190	450–500
Beginning pathology	120	40	20	3	4	45	200	Less than 400 or more than 550
Anemia, primary	100–*300*	40–*100*	20–70	2–*3*	4–*10*	50–70	150–160	400–500
Bichloride poisoning	110–*300*	*100–350*	70–*300*	*5–35*	*10–15*	*45–20*	*200–350*	*500–700*
Diabetes, mild	*140–300*	25–35	10–15	1–2	1–3	*60–30*	170–*250*	450–550
Diabetes, severe	*300–1200*	*30–40*	15–*20*	2–4	*4–10*	50–*10*	*300–800*	400–*600*
Diabetes, renal	70–95	25–35	10–15	1–2	1–3	55–75	170–*210*	450–500
Eclampsia	110–*180*	35–*60*	6–*25*	1–3	4–11	55–*12*	200–230	450–600
Gout	70–110	25–35	10–15	1–2	*4–10*	50–70	150–200	450–550
Intestinal obstruction	80–120	*75–170*	*40–150*	*3–10*	5–10	*45–25*	150–200	400–500
Nephritis, Hemorrhagic Initial	100–140	*40–60*	*20–40*	*2–4*	*4–12*	60–*30*	170–200	450–600
Acute	110–*180*	*60–150*	*30–100*	*3–6*	*5–15*	*45–20*	*170–250*	450–600
Chronic	110–*160*	*40–100*	*20–70*	2–4	*4–10*	70–40	170–*350*	450–*750*
Terminal	110–*200*	*100–350*	*60–300*	*5–35*	*10–27*	40–12	*200–300*	360–600
Nephritis, Degenerative	110–*180*	*60–100*	*20–60*	2–4	2–5	50–*25*	300–*500*	500–600
Arteriosclerotic	110–140	*40–60*	*20–30*	2–3	*3–10*	70–45	*200–250*	400–600
Nephrosis, Primary (Lipoid)	70–110	Albumin-globulin ratio reversed (Normal = 1.7/1 to 3/1) 25–35	10–15	2–4	3–5	60–30	170–250	450–500
Secondary (Glomerular)	70–110	Albumin-globulin ratio—not necessarily reversed 40–100	25–40	2–4	3–5	60–30	170–300	450–500
Pneumonia, lobar	100–*180*	*40–80*	20–*50*	2–3	4–10	*40–50*	150–200	350–450
Uremia	110–*200*	*100–400*	*60–330*	*5–35*	*10–25*	*25–5*	170–*350*	450–650
Prostatic obstruction	100–150	*25–70*	12–*40*	2–4	*3–9*	*60–35*	150–190	500–600
Polycystic kidney, bilateral	*140–200*	*60–120*	*23–75*	*3–8*	*5–10*	*50–30*	150–200	450–600

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Reviews and papers dealing with blood coagulation are the following: Quick, The *Hemorrhagic Diseases* (1942), Howell, *J. Am. Med. Assoc.*, **117**, 1059 (1941) (review); Chargaff, *Adv. Enzym*, **5**, 31 (1945) (review), Ferguson, *J Laboratory and Clinical Medicine*, **24**, 273 (1938) (a standardized laboratory procedure for the study of coagulation); Brinkhous, *Medicine*, **19**, 329 (1940) (a review of prothrombin and vitamin K, see, also vitamin K); Quick, *Am. J. Physiol*, **131**, 455 (1940) (calcium in blood coagulation); Mertz, Seegers and H P. Smith, *Proc Soc Exp Biol. Med.*, **42**, 604 (1939) (interrelationship of prothrombin, thromboplastin and thrombin); Chargaff, Bendich and Cohen, *J. Biol Chem.*, **156**, 161 (1944).

Seegers, Loomis and Vandenbelt, *Arch. Biochem*, **6**, 85 (1945), claim to have isolated prothrombin in the pure state.

Quick, *Am. J. Physiol.*, **140**, 212 (1943), indicates that prothrombin is composed of calcium and two separate components.

Loomis and Seegers, *Arch. Bioch.*, **5**, 265 (1944), are the authors of a paper dealing with the factors which influence the activation of prothrombin.

Ferguson discusses his new theory of blood clotting in *Science*, **97**, 319 (1943).

Various substances, from chloramin-T to ninhydrin, coagulate fibrinogen; see Chargaff and Bendich, *J. Biol. Chem*, **149**, 93 (1943).

The use of a phenol-formaldehyde resin to prevent blood from clotting is described by Steinberg, *Proc Soc. Exp. Biol. Med.*, **56**, 124 (1944).

For articles on anticoagulants, see Prandoni and Wright, *Bull N. Y. Acad. Med*, July, 1942, p. 433 (heparin and the coumarin derivative); Jorpes and Bergstrom, *J. Biol. Chem.*, **118**, 447 (1937) (heparin), Crafoord and Jorpes, *J. Am. Med. Assoc.*, **116**, 2831 (1941) (heparin in thrombosis); Ziff and Chargaff, *J. Biol. Chem.*, **136**, 689 (1940) (mode of action of heparin); Best, *Harvey Lectures* (1940–41), p. 66 (heparin and thrombosis); Charles and Todd, *Biochem. J.*, **34**, 112 (1940) (chem. of heparin).

Link's pioneer work on the anticoagulant from spoiled sweet clover hay (dicoumarol) is described in *Harvey Lectures* (1943–44), 162. See, also, Link, Overman, Sullivan, Huebner and Scheel, *J. Biol Chem.*, **147**, 463 (1943) (salicylic acid and hypoprothrombinemia); Overman, Stahlman, Huebner, Sullivan, Spero, Doherty, Ikawa, Graf, Roseman and Link, *Ibid*, **153**, 5 (1944) (anticoagulant activity in the 4-hydroxycoumarin group).

For clinical applications of dicoumarol, see Allen, Barker and Waugh, *J. Am. Med. Assoc*, **120**, 1009 (1942); Wright and Prandoni, *Ibid*, **120**, 1015 (1942). Bollman and Preston, *Ibid*, **120**, 1021 (1942); Butsch and Stewart, *Ibid*, **120**, 1025 (1942).

Heparin and dicoumarol as anticoagulants are reviewed by Quick in *Physiol. Rev.*, **24**, 297 (1944).

For the chemistry of heparin, see Wolfrom, Weisblat, Karabinos, McNeely and McLean, *J. Am. Chem. Soc.*, **65**, 2077 (1943); Kuizenga and Spaulding, *J. Biol.*

Chem, **148,** 641 (1943); Wolfrom and Karabinos, *J. Am. Chem. Soc*, **67,** 679 (1945).

The important problem of blood transfusion therapy is discussed by Kolmer and Tuft, *Clinical Immunology, Biotherapy and Chemotherapy* (1942), p 244. Cohn, *American Scientist*, **33,** 61 (1945) is the author of an excellent review dealing with the work of the fractionation of plasma See, further, *Nutr. Rev*, **3,** 103 (1945); Janeway, *Bull N. Y. Acad. Med*, April, 1945, p. 202 (use of serum γ-globulin in measles), Cohn, *Science*, **101,** 51 (1945); Janeway, *J. Am Med. Assoc*, **126,** 674 (1944) (albumin in shock and hypoproteinemia; γ-globulin in measles), Ingraham and Bailey, *Ibid*, **126,** 680 (1944) (products of fibrinogen and thrombin in surgery); DeGowin, *Ibid*, **127,** 1037 (1945) (whole blood transfusions), Stenerson, *Chemical and Engineering News*, **23,** 714 (1945) (processing of blood plasma), *Bulletin of the Lederle Labs*, **12,** 47 (1944).

For the chemistry of the substance in pernicious anemia, see Cohn and associates, *J. Biol Chem*, **77,** 325 (1928); Dakin, Ungley and West, *Ibid*, **115,** 771 (1936), Jacobsen and Subbarow, *J. Am. Med. Assoc*, **116,** 367 (1941). The clinical phases of this disease are discussed by Minot, Murphy and Stetson, *Harvey Lectures*, Ser. 23 (1927–1928), p. 154, Castle, *Ibid.*, Ser. 30 (1934–1935), p. 37; Murphy and Howard, *J. Am. Med. Assoc*, **112,** 106 (1939); Askey, *Ibid*, **117,** 907 (1941), Erdos, *Science*, **96,** 141 (1942).

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Evidence for the liver as the probable site of formation of blood albumin, fibrinogen and prothrombin is presented by Post and Patek, *Bull. N. Y. Acad. Med.*, Dec. 1943, p. 815.

These are two stages in the formation of fibrin from fibrinogen, claims Robbins, *Am. J. Physiol.*, **142,** 581 (1944)

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CHAPTER 14

IMMUNOCHEMISTRY. CHEMOTHERAPY

IMMUNOCHEMISTRY

Micro-organisms Which Cause Disease.—These may be divided into three groups: animal parasites, such as protozoa; vegetable parasites, such as bacteria and fungi; and filter-passing viruses.

Amebic dysentery, malaria and sleeping sickness—all three common in tropical countries—are caused by protozoa. Since the spirochetes are usually classed as protozoa, syphilis would be included here.

In temperate climates the chief diseases are caused by bacteria. Some of these bacteria and the diseases they give rise to are the following:

Streptococcus pyogenes, or β-hemolytic streptococcus, includes some thirteen different strains of bacteria which give rise to septic wounds, blood-poisoning, erysipelas, scarlet fever, etc.

Diplococcus pneumoniae, or pneumococcus, includes some thirty-four strains which are the common cause of pneumonia.

Staphylococcus aureus, commonly found in the skin, usually causes boils.

Neisseria gonorrhoeae, or gonococcus, gives rise to gonorrhea.

Neisseria intracellularis, or meningococcus, causes cerebrospinal meningitis.

Various bacteria also cause diphtheria, tuberculosis, cholera, plague, lock-jaw, anthrax, etc.

Diseases due to viruses—particles which resemble bacteria but, unlike them, pass through unglazed porcelain—are smallpox, infantile paralysis, typhus fever, common colds, and probably measles, mumps, influenza, etc.

So far, greater success has attended the treatment of protozoal and bacterial diseases than the virus diseases.

Defenses Set Up By the Body.—The skin and the acid of the stomach are among these defenses—the skin by preventing entrance of bacteria and the hydrochloric acid by destroying them. But many bacteria do get into the body. The large intestine (and the eliminated feces) is full of bacteria, but these cannot penetrate the intestinal wall to enter the body proper.

However, whether through the nose, through the throat or through a cut, or as a result of "lessened resistance," bacteria do penetrate the blood and tissues. To counter this menace, certain cells, macrophages, engulf the bacteria and are usually strong enough to destroy them. The macrophages are found lining the blood vessels of the liver, bone marrow, etc. ("reticulo-endothelial system") and some wander and

may even circulate in the blood. The white cells (leukocytes) of the blood have a function similar to the macrophages.

Immunochemistry.—We have seen how the body handles certain toxic substances by detoxicating them and then eliminating the detoxified products (Chap. 11). When, however, the toxic substance which enters the blood stream is chemically of a sufficiently complex nature—approaching, say, that of a protein in complexity—the body tries to resist the effects of such toxicity by building up for its defense "antibodies" which tend to offset the effects of the toxic substance.

The substances which give rise to these antibodies are called "antigens." The antigens include bacteria, protozoa, molds, and "foreign" proteins.

In general, proteins which are "foreign" to the body—that is to say, proteins which are not normally found in the serum of the animal—give rise to antibodies. Certain polysaccharides may also stimulate antibody production.

So far, all attempts to prepare antibodies free from protein have been unsuccessful.

When a suspension of living or dead bacteria (pneumococci, for example) is injected into an animal, an "immunity" to further infection by subsequent injection of bacteria may be acquired, and the serum of the animal reacts characteristically when mixed with an extract of the bacteria. The characteristic reaction resulting from the mixing of the serum with the bacteria originally used for the injection may give rise to a clumping or "agglutination," to a dissolving or "lysis," or to precipitate, "precipitin," depending upon conditions. The "agglutinins," "lysins," and "precipitins" are all antibodies.*

The antigenic properties of the bacteria are usually ascribed to the proteins which they contain. Proteins, in general, with the notable exception of gelatin, give rise to antibodies; and these substances are removed from the serum with the fraction containing the globulins—a fact which is used as evidence that the antibodies may resemble the serum globulins.

These antibodies, then, are in all probability globulins, but differ from the ordinary serum globulins in one important respect: their reactivity with the antigen; which means that the antibody molecule differs in some structural way from the serum globulins.

The specificity of these immunological reactions is remarkable. The immunological test for human blood has already been given (see p. 272). Here is another example: if crystallized egg albumin is injected, the

* Heidelberger, and also Marrack, consider specific immune precipitation and specific agglutination of bacteria as due to the combination of antigen and antibody in such a way that molecules of antigen may become attached to molecules of antibody through one or more linkages.

As this concept postulates several reactive groupings in the molecules, an antigen-antibody complex may combine with other antigen or antibody molecules, or with preformed antigen-antibody combinations to build up large aggregates which separate from solution (or, in the case of bacteria, clump together and settle).

The process of immune combination is a reversible one. Aggregates may dissociate into uncombined antigen and antibody molecules. This dissociation, however, is relatively small.

antibodies produced, present in the serum, will *not* precipitate solutions of another albumin, such as crystalline horse serum albumin *

With the help of the electron microscope, we can actually visualize the antigen-antibody reaction. Tobacco mosaic virus was selected as the antigen. The result of the injection in the rabbit caused the serum of the animal to develop the corresponding antibody. The rabbit antiserum was the source, then, of the antibody.

In (1), Fig. 73, the microphotograph shows the suspension containing 0 01 mg. of tobacco mosaic virus per cc The molecules stand out sharply and have widths of about 15 mμ and lengths of about 280 mμ.

Figure 73, (2), is a microphotograph of a mixture of tobacco mosaic virus and normal rabbit serum diluted 1:100 with distilled water. The tobacco mosaic virus molecules stand out sharply with the normal lengths and widths, and with but few particles from the serum adsorbed on them. The contaminating bacterium which may be observed in this figure serves to give a good idea of the relative size of the particles of tobacco mosaic virus.

Figure 73, (3), is a microphotograph of the mixture of tobacco mosaic virus and antiserum diluted 1:100, which had stood for one hour. The particles of tobacco mosaic virus appear at much greater contrast and are three or four times as wide as in the former preparations. This indicates that particles from the antiserum have become attached to the tobacco mosaic virus molecules, making them appear wider and at the same time presenting a thicker specimen to the electron beam.

Figure 73, (4), shows a typical portion of the preparation of tobacco mosaic virus plus antiserum after standing for several hours at a dilution of 1:100. The particles are still extremely thick.

Pauling has produced substances *in vitro* which simulate the action of antibodies. "It is assumed," writes Pauling, "that antibodies differ from normal serum globulin only in the way in which the two end parts of the globulin polypeptide chain are coiled; these parts, as a result of their amino-acid composition and order, having accessible a very great many configurations with nearly the same stability. Under the influence of an antigen molecule the parts assume configurations complementary to surface regions of the antigen, thus forming two active ends. After the freeing of one end and the liberation of the central part of the chain, this part of the chain folds up to form the central part of the antibody molecule."

Globulin, therefore, in the presence of the antigen, was subjected to temperature changes over a period of days.

In one experiment, a solution of 1 per cent pneumococcus poly-

* In anaphylaxis, or allergy, it is assumed that "allergens" are the "substances in the diet or in the environment that act as specific incitants of a distinct group of human disease reactions called allergies"; and that these allergens are antigens, or closely related to them, and therefore also probably proteins.

Spies, Coulson, Bernton and Stevens have isolated potent allergenic protein fractions [*J. Am. Chem. Soc.*, **62**, 1420 (1940)]. See, also Spies and Coulson, *Ibid.*, **65**, 1720 (1943).

Northrop has also purified and crystallized diphtheria antitoxin, a protein, which is precipitated by diphtheria toxin [*J. Gen. Physiol.*, **25**, 465 (1942)].

saccharide type III and 1 per cent bovine γ-globulin was held at 57° for fourteen days. "This temperature seems to be high enough to cause the protein chains to unfold and to refold, under the influence of the antigen, into specific complementary configurations."

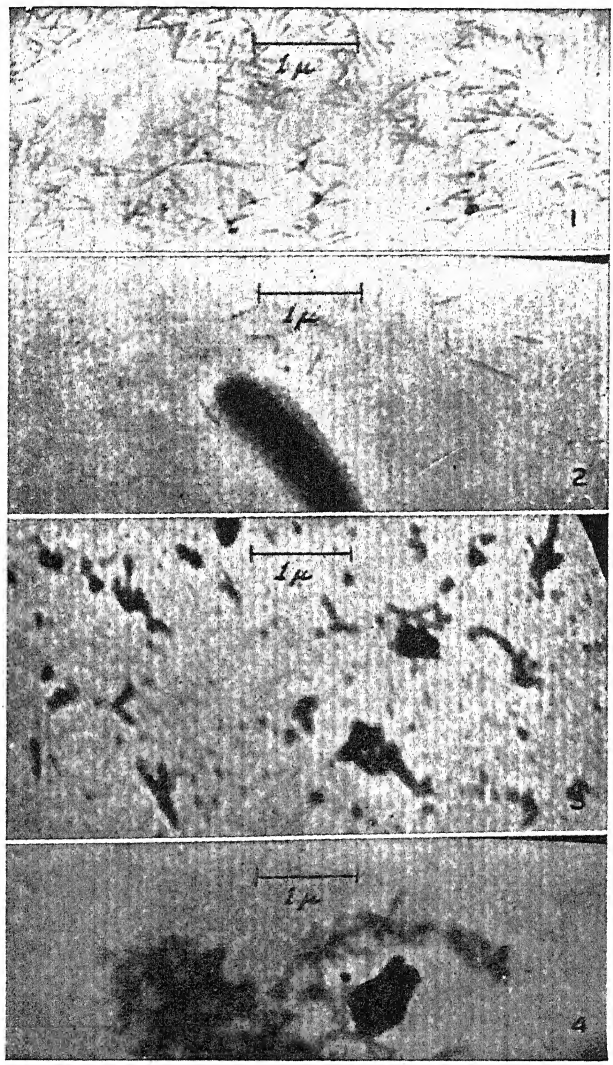


Fig. 73.—Antigen-antibody reaction. (Anderson and Stanley, *J. Biol. Chem.*, 139, 344.)

The resulting solution proved a specific antiserum in that it precipitated type III polysaccharide but not types I or VIII, and agglutinated pneumococci type III but not types I or II.

CHEMOTHERAPY

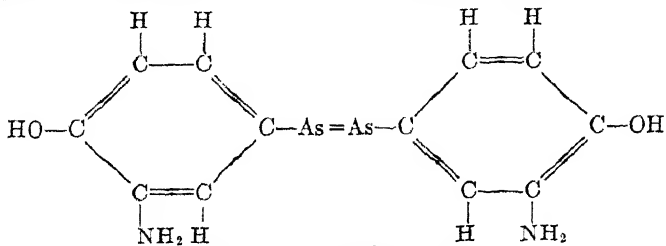
Aside from the defense mechanisms of the body for protecting itself against the effects of bacterial invasion, other possibilities sug-

gest themselves. One method is to employ heat. Sufficient heat destroys bacteria. It is obvious, however, that it is practically impossible to increase the temperature of the body to a degree necessary to destroy the organisms without harming the tissues. The same applies to the action of many chemicals. Phenol, formaldehyde, iodine, mercuric chloride, chloride of lime, etc., are all excellent disinfectants, but they are also effective destroyers of protoplasm.

The problem, then, was to find chemicals, drugs, which would destroy the organisms without materially harming the tissues. This is the problem of chemotherapy.

A partial answer to this problem was obtained when it was shown that malaria could be cured, to some extent, at least, with quinine, and syphilis with mercury compounds. This work also suggested that a definite chemical substance was "specific" in its attack on a definite organism.

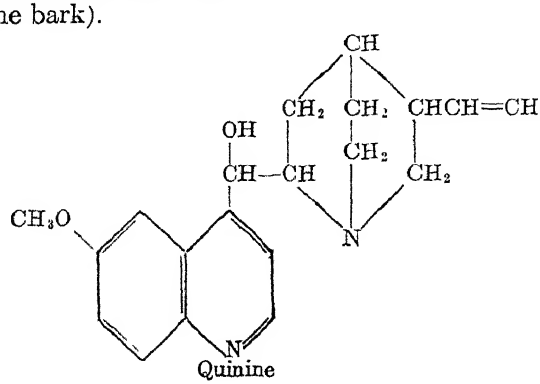
The triumph of chemotherapy may be said to date back to 1910 when Ehrlich discovered "606" or salvarsan as a cure for syphilis.



Salvarsan or arsphenamine.
(3,3'-Diammo-4,4'-dihydroxyarsenobenzene.)

From 1910 on rapid strides were made in chemotherapy; but, until 1935, practically all of the successful chemical substances in use were remedies for tropical diseases caused by protozoa.

For example, one of the most striking of these diseases for which chemotherapy found an answer was malaria, a disease caused by the protozoan, *Plasmodium*, transmitted by the female *Anopheles* mosquito to man. It had been known for a long time that the bark of the cinchona tree was a remedy for malaria, but it took years to isolate the important alkaloid responsible for the cure: quinine (one of twenty odd alkaloids present in the bark).

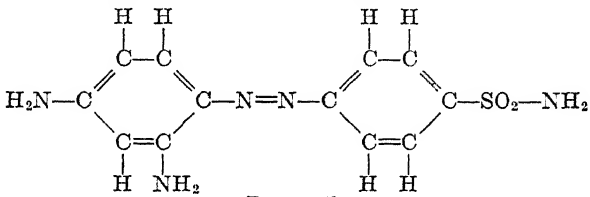


Quinine

This valuable drug is obtained largely from the Dutch East Indies. During the Second World War the occupation of these islands by the Japanese made it difficult for American health authorities to supply our troops with the needed quinine. Synthetic substitutes, such as plasmochin (a quinoline derivative), and atebrin (an acridine derivative) came into use.

The synthesis of quinine itself, for years a stumbling block to organic chemists, was accomplished by Woodward and Doering in 1944. It was not until 1935, with the use of sulfanilamide, that the first of a series of substances was used which was effective against bacteria—the bacteria which give rise to the commonest diseases (p. 278).

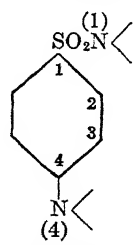
Sulfonamide Compounds.—Domagk, in an extension of the application of dyes to chemotherapy, discovered that prontosil injected into mice infected with streptococci had a definitely curative effect.



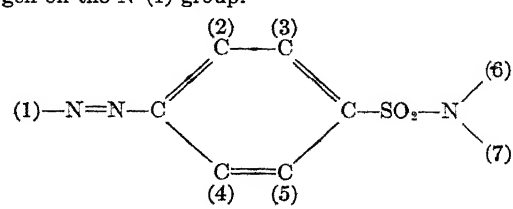
Prontosil.
(4-Sulfonamido-2′,4′-diaminoazobenzene.)

Further work made clear that prontosil was an effective therapeutic agent against the β-hemolytic streptococcus (p. 278), an agent which destroys red blood cells, or which gives rise to red rashes on the skin (erysipelas and scarlet fever). It was also apparent, judged by the use of hundreds of different substances, that the most effective skeleton type was the following (see footnote) with various additional groups attached to positions (1) and (7).*

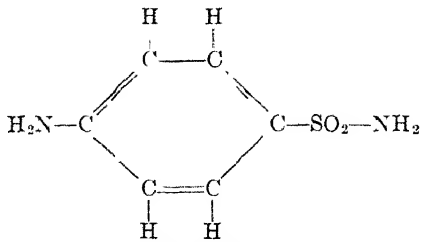
* Writing the skeletal form of sulfanilamide thus:



Where the N in the *p* position is in position 4, and the other N would therefore be in position 1, we may say that the best results so far obtained are from substitution of hydrogen on the N (1) group.

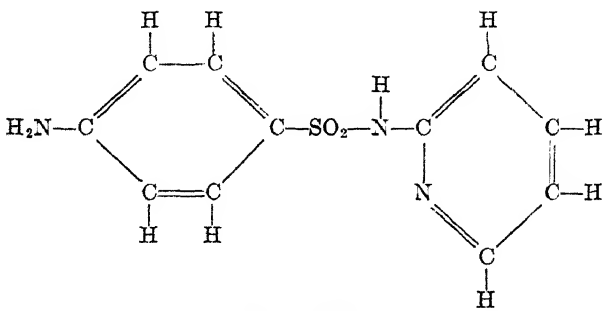


It was next shown that prontosil was converted in the body into sulfanilamide, a very well known organic substance; and that **sulfanilamide** itself was most potent.

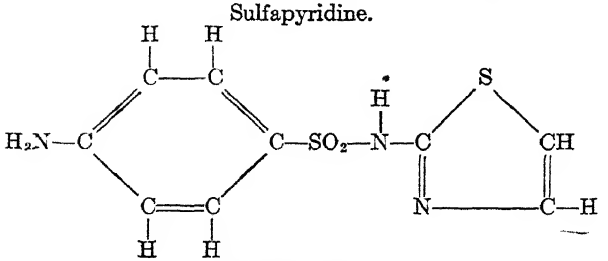


Sulfanilamide.
(*p*-Aminobenzenesulfonamide.)

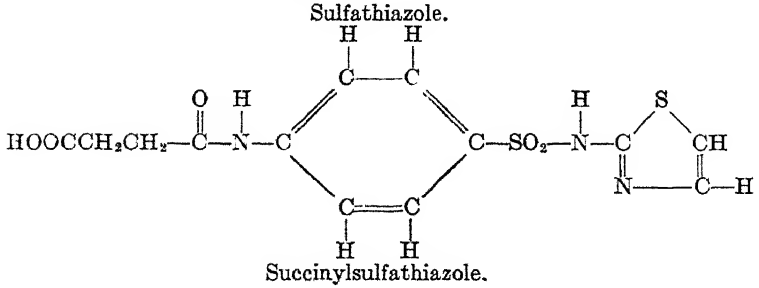
Extremely effective compounds were obtained by replacing the hydrogen in position (7) with pyridine, thiazole, and other groups (see p. 286):



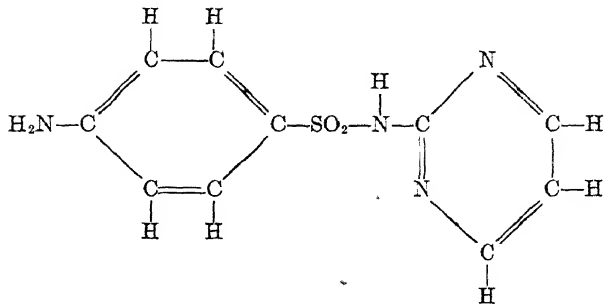
Sulfapyridine.



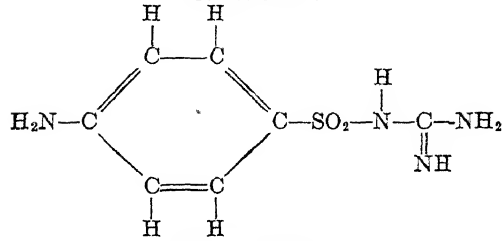
Sulfathiazole.



Succinylsulfathiazole.



Sulfadiazine.



Sulfaguanidine.

The steps in the synthesis of some of these sulfonamides are graphically shown in the following diagram (Fig. 74):

Clinical Use.—The sulfonamide drugs are administered by mouth and in fairly large quantities. A gram may be given every three to four hours and the dosage continued for five to ten days during the critical period of the illness.

One or more of the drugs have been used, sometimes with amazing success, in septic wounds and blood poisoning, puerperal fever (disease of childbirth), erysipelas, meningitis, malignant endocarditis (infection of the heart), pneumonia, gonorrhea, certain kidney infections, etc.

But quite a number of bacterial diseases exist which, apparently, are not influenced by the "sulfa drugs." Among them are tuberculosis, typhoid, paratyphoid, cholera, bacillary dysentery and whooping-cough.

Also the main body of virus diseases are not influenced by treatment with the sulfonamides. These include smallpox, measles, infantile paralysis, colds and influenza.

Mode of Action.—An antisulfanilamide factor, obtained from an extract of yeast, was traced to *p*-aminobenzoic acid (p. 169). Woods showed that a concentration as low as 1:10,000 ($5.8M \times 10^{-8}$) was active in partially inhibiting the bacteriostatic effect of sulfanilamide at a concentration of 1:20,000 ($3M \times 10^{-4}$).

This has suggested to Woods and to Fildes a possible mechanism of the action of sulfonamide compounds. *p*-Aminobenzoic acid is regarded as an essential compound synthesized by the bacterial cells from amino acids, or obtained by the cells from their environment. To be properly utilized by the cell, the *p*-aminobenzoic acid is acted upon

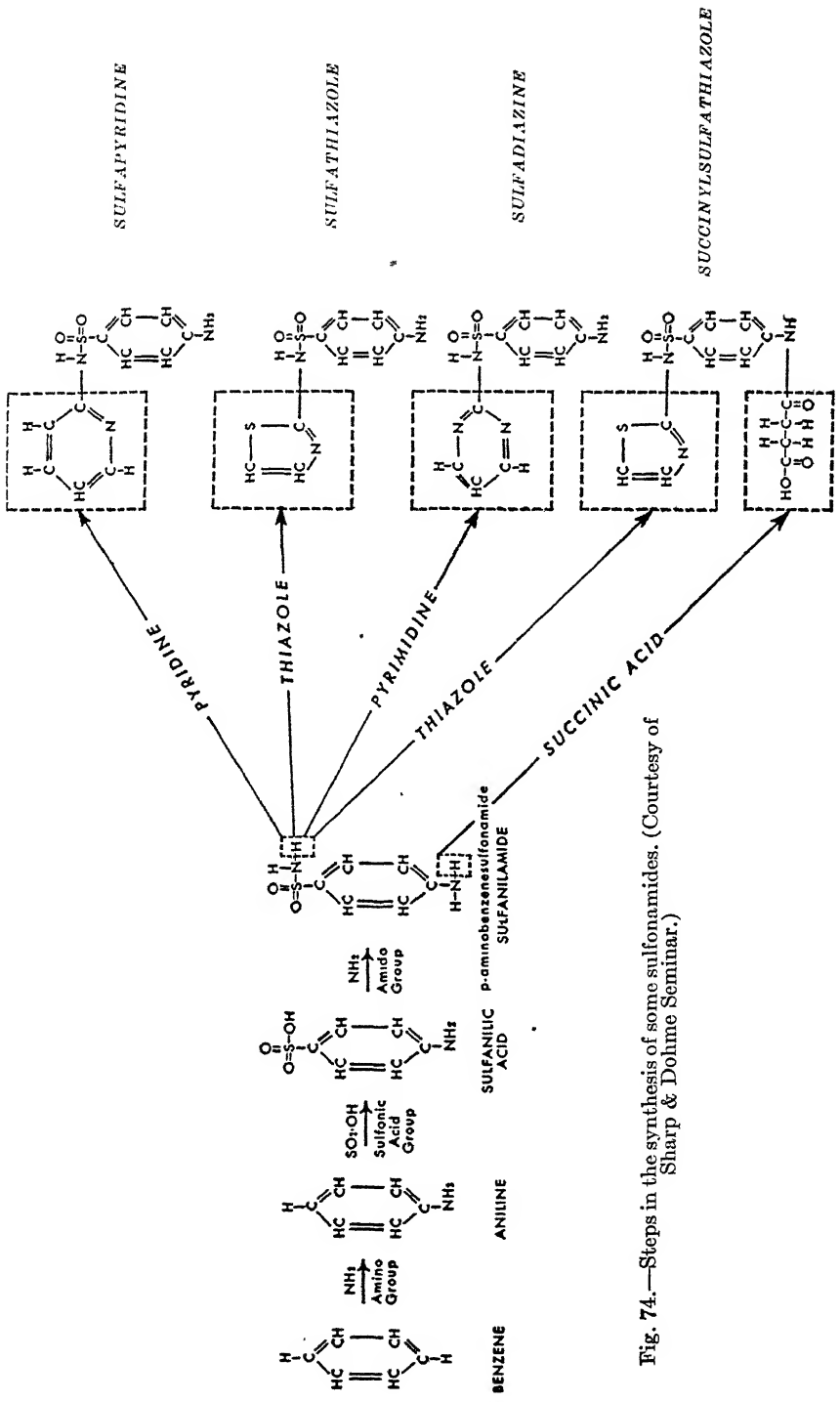
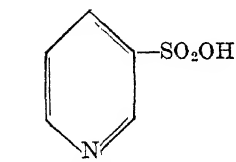


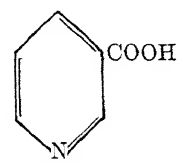
Fig. 74.—Steps in the synthesis of some sulfonamides. (Courtesy of Sharp & Dohme Seminar.)

by an enzyme. If sulfanilamide is present, it also is acted upon by the enzyme and thereby prevents the *normal* metabolism of the bacterial cell. The bacteria fail to get their proper nutrition and are ultimately destroyed.

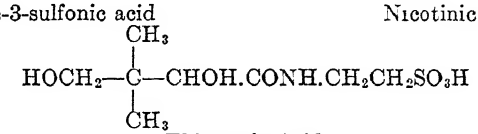
These findings of Woods and others have led to some very suggestive work with regard to the relationship of chemical structure to physiological action. For example, pyridine-3-sulfonic acid, which is related to nicotinic acid in a manner similar to the relationship of sulfanilamide to *p*-aminobenzoic acid, inhibits bacterial growth, and its corresponding vitamin analog restores this growth. The same is true with thiopanic acid (pantoyltaurine), the sulfur analog of pantothenic acid.



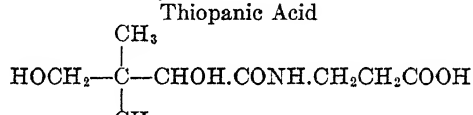
Pyridine-3-sulfonic acid



Nicotinic acid



Thiopanic Acid



Pantothenic Acid

The possibilities spread still further. Woolley and others have shown that, in some cases, by using compounds, modified in structure, in the place of the normal (vitamin) compounds, specific vitamin deficiencies may be produced. For example, isoriboflavin (the 5,6-dimethyl modification), inhibits the growth-promoting action of riboflavin (the 6,7-dimethyl form; see p. 153).

All the evidence points to the conclusion that the sulfonamides act by preventing the growth of bacteria (bacteriostasis) rather than by destroying them.*

Excretion.—The sulfonamides are detoxicated in various ways in the body, presumably in the liver. They are excreted through the kidneys and appear in the urine in one or more of the following modi-

* Cowles has shown that the sulfonamide activity increased as the acid pK of the drug decreased toward 7, but that as the acid strength increased from then on, the activity diminished. Bell and Roblin obtained maximum potency with the drugs at a *p*H of 7.

On the assumption that sulfonamide activity is due to the blocking of an enzyme system (containing protein, of course), Klotz concludes that inhibition of bacterial growth is due to a reversible combination between the basic form of the drug (the anion) and the enzyme molecule (protein), and that the law of mass action can be applied. The reversal of sulfonamide bacteriostasis by the addition of *p*-aminobenzoic acid is considered from the same point of view (Klotz, *J. Am. Chem. Soc.*, **66**, 459 (1944)).

fications: as an N_4-acetyl derivative (see, p. 245); as a monohydroxyl derivative; as a glucuronide; as an ethereal sulfate.

Intestinal Antiseptics.—Sulfaguanidine and succinylsulfathiazole are very poorly absorbed; they have therefore been used as intestinal antiseptics.

Experiments on animals have shown that these sulfa compounds, by destroying organisms in the intestine, often prevent, to a large extent, the synthesis of a number of vitamins which are normally brought about with the help of these organisms. Some of these vitamins are vitamin K and a few belonging to the vitamin B complex (biotin, folic acid, *p*-aminobenzoic acid).

TABLE 42.—SUMMARY OF CHEMICAL AND BIOLOGICAL PROPERTIES OF SOME OF THE MORE IMPORTANT ANTIBIOTIC SUBSTANCES. (Waksman, *Am. J Public Health*, **34**, No. 4.)

Substance.	Organism.	Active against bacteria	Properties.
Actinomycin A	*A. antibioticus*	Selective quantitative action	Soluble in ether and alcohol, not in petrol ether, orange colored, highly toxic, thermostable, nitrogen bearing ring compound
Aspergillic acid	*A. flavus*	Active against both Gram-positive and Gram-negative bacteria	Soluble in alcohol, ether, acetone, not in petroleum ether. Acid nature. m p about 96° C, m w. 224, about 13 per cent nitrogen.
Citrinin	*P. citrinum*	Non-selective.	Soluble in water and in alcohol, precipitated by acid, quinone.
Clavacin	*A. clavatus*	Active against Gram-negative and some Gram-positive bacteria. Highly bactericidal	Soluble in ether, chloroform, alcohol and water. Toxic.
Flavicin	*A. flavus*	See penicillin.	Similar in all respects to penicillin.
Fumigatin	*A. fumigatus*	Largely active against Gram-positive bacteria.	White, needle-shaped crystals, m p. 185°–187°; soluble in alcohol, limited solubility in water.
Gliotoxin	*Trichoderma, Gliocladium*	Non-selective; fungicidal and bactericidal.	Soluble in chloroform, benzol alcohol, sparingly in water, contains nitrogen and sulfur.
Gramicidin	*B. brevis*	Lytic to Gram-positive bacteria.	Soluble in ether and acetone, thermolabile; active *in vivo*, polypeptide
Notatin	*P. notatum* and *P chrysogenum*	On Gram-positive and Gram-negative bacteria.	Insoluble in organic solvents, soluble in water; acts, in presence of glucose.
Penicillic acid	*P. puberulum*	Active against Gram-positive and also upon Gram-negative bacteria.	Colorless, soluble in water.
Penicillin	*P notatum* and *P. chrysogenum*	Acts largely upon various Gram-positive aerobic and anaerobic bacteria.	Soluble in alcohol and water; thermolabile; active *in vivo;* low toxicity.
Proactinomycin	*Pr. gardneri*	Acts primarily upon Gram-positive bacteria.	Soluble in ether, benzene and water. Toxic.
Pyocyanase	*Ps. aeruginosa*	Lytic action on many Gram-positive and Gram-negative bacteria.	Thermostable; lipoid, actively largely due to unsaturated fatty acids.
Pyocyanin	*Ps. aeruginosa*	Bactericidal action largely against Gram-positive bacteria.	Chloroform-soluble, blue pigment, thermostable.
Streptothricin	*A. lavendulae*	Active on various Gram-negative and some Gram-positive bacteria.	Soluble in water and in acid alcohol, not in ether, organic base; thermostable. Low toxicity; active *in vivo.*
Tyrocidine	*B. brevis*	Lytic to Gram-positive and Gram-negative bacteria.	Soluble in alcohol, not in ether; thermostable; polypeptide.

Antibiotic Substances.—These substances, antimicrobial in action, are found in molds, yeasts and bacteria. They inhibit the growth of bacteria—they are bacteriostatic—though they may also, and to a lesser extent, show bactericidal properties. These substances show a certain specificity in their action. Some act on Gram-positive bacteria

and show little action upon Gram-negative ones. Others show selective action on some of each group.

Despite the fact that a few of these antibiotic substances have been crystallized, little is known of the chemical structure of most of them. Notatin is an exception, since it has been well established that the substance is a flavoprotein. Also, citrinin, penicillic acid and fumigatin are probably compounds belonging to the quinone group.

A list of some of these antibiotic substances is given in Table 42.

So far, the only one of these substances which has found wide therapeutic use is penicillin; and this very largely because it is nontoxic to man while preventing the growth of a wide number of pathogenic organisms.

Fig. 75.—Photomicrograph (× 100) of penicillin-producing mold growing on agar. Branching ends of conidiophores give genus its name of *Penicillium*, from the Latin for brush. (*Penicillin*, Abbott.)

Penicillin.—Florey records that as far back as 1877 Pasteur noticed that the growth of one type of bacteria may be stopped by the simultaneous growth of another. Later this phenomenon was shown to be due to the production of some definite chemical substance by the antagonistic microorganism. These chemical inhibitors were called *antibiotic*.

In 1929 Fleming made his great discovery while studying the growth and properties of staphylococcus. He grew the organism on a solid medium containing agar. He noticed that "in a large colony of a contaminating mold the staphylococcus colonies became transparent and were obviously undergoing lysis." This observation, due to the accidental contamination of the media by bacteria and molds from the air, was the key to the discovery.

The contamination, in Fleming's case, was due to a mold colony. He cultivated the mold in liquid broth, and noticed that during growth something appeared which inhibited the growth of some organisms.

Fleming called this "something" *penicillin*, for the mold was identified (somewhat later) as *penicillium notatum*—a mold not at all common and not the one found on bread (Fig. 75).

Fleming made the important observation that his extract containing penicillin was not poisonous to animals and did not harm white blood cells.

It was not until 1938 that this work of Fleming's was actively pursued, this time by Florey and his associates. This group developed a quick assay method and uncovered several chemical properties of penicillin, such as, that it exhibited acidic properties, was unstable in acid and alkaline media, and more stable in a neutral medium.

Florey and his associates found that the penicillin brew, after acidification, could be extracted with ether, and that the ether extract (containing much of the penicillin) when mixed with some water and the right amount of alkali, yielded a more stable alkaline salt.

In investigating the effect of penicillin on bacteria, the group headed by Florey found the following to be susceptible: *Streptococcus pyogenes*, causing pus formation; *Staphylococcus aureus*, associated with bone diseases and boils—so important in war; and bacilli giving rise to pneumonia, diphtheria gas gangrene, gonorrhea and meningitis. The penicillin had no effect on organisms associated with plague, cholera, dysentery and tuberculosis.

The properties of penicillin which make it so valuable a remedy for man were also largely uncovered by the Florey group. They confirmed Fleming's observation that the substance was non-toxic to animals and to white blood cells and tissue cultures (body cells grown outside of the body). They further found that the activity of penicillin was not affected by pus, blood or breakdown products of dead tissue (which is not true of the sulfonamides), and that it was little affected by the number of bacteria present (also not true of the sulfonamides).

Florey found that the penicillin was destroyed if given orally, since the substance is attacked by the acid of the stomach.* He also observed that the material was very rapidly excreted, which meant that comparatively large, and repeated, doses were necessary.†

How did the substance act? Florey believed that it was largely bacteriostatic in its action—that it stopped the growth of bacteria and then gave the white blood cells an opportunity to ingest and kill them.

Florey relates one of the early tests of the substance on mice, in preparation of its application to man. Mice were inoculated with germs which normally would kill them. "We sat up all night injecting penicillin every three hours into the treated group; and I must confess

* Some encouraging results have been obtained, however, when penicillin was mixed with fat or given in form of capsules (*J. Am. Med. Assoc.*, **127**, 1129 (1945).

† Here, too, delayed excretion is possible by giving *p*-aminohippuric acid, for example. See Bronfenbrenner and Cutting, *Science*, **101**, 673 (1945).

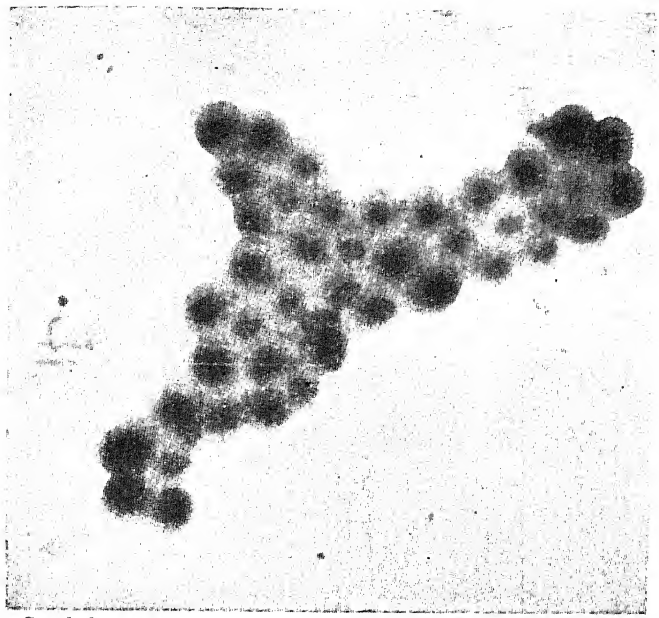


Fig. 76.—*Staphylococcus aureus* prior to influence of penicillin: (reduced from original electron micrograph, 38,000 ×). (*Courtesy Radio Corporation of America.*)

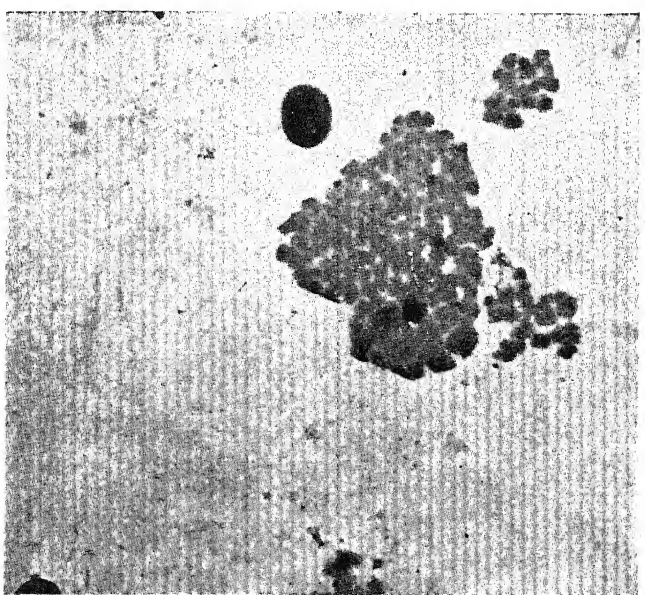


Fig. 77.—Penicillin action on *Staphylococcus aureus:* (reduced from original electron micrograph, 38,000 ×). (*Courtesy Radio Corporation of America.*)

it was one of the more exciting moments when we found in the morning that all the untreated mice were dead and all the penicillin-treated ones were alive."

"The discovery of penicillin," concludes Florey, "was one of the luckiest accidents . . . for without exception, all other mold antibiotics so far examined are poisonous."

The affect of penicillin on bacteria is graphically illustrated in Figs. 76 and 77, which represent photographs taken with the electron microscope.

Assay.—The "cup method," devised by Florey and associates, is widely used. An agar plate is seeded with cultures of the test organ-

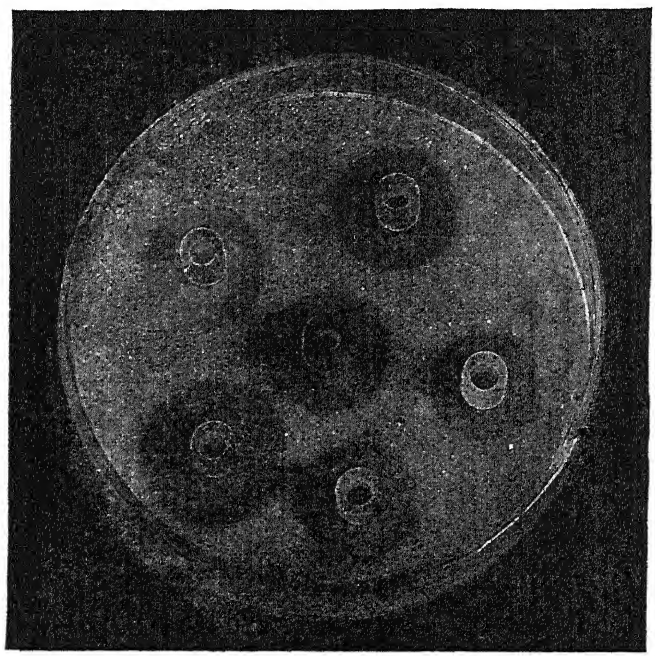


Fig. 78.—Potency is indicated by inhibitions of bacterial growth (the clear zones) around the five cylindrical cups set on the agar plate and containing penicillin. (*Therapeutic Notes*, March, 1944, Parke, Davis & Company.)

isms. Cylinders of glass, short and open at both ends, are placed on the agar, and the solutions which are to be tested are placed in the cylinders. After incubation, where the penicillin has diffused out and inhibited growth there appears a circular and "clear" zone around each cylinder. The diameter of the zone is related to the concentration of the penicillin (Figs. 78, 79).

The "Florey" or "Oxford" unit, widely used, is defined as "that amount of penicillin which, when dissolved in 50 ml. of meat extract broth, just inhibits completely the growth of the test strain of *Staphylococcus aureus.*"

It has been suggested that an International Unit be adopted, based on the activity of the crystalline calcium salt of penicillin. The

unit selected is 0.6 micrograms (μg) of this salt. This has been generally agreed upon.

Chemistry.—Several antibioties of the type of penicillin are known. The general empirical formula is $C_9H_{11}O_4SN_2.R$. In one of them, F-penicillin, R = $-CH_2.CH = CH.CH_2.CH_3$. The structural formulas proposed show them as thiazole derivatives. Crystalline preparations of the substance (which shows acidic properties) in the form of sodium and calcium salts, have been prepared.*

Clinical Use.—Penicillin has been used extensively, and with marked success, in the following: infections due to staphylococci

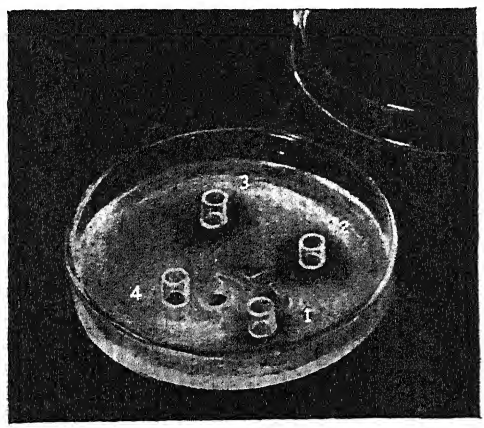


Fig. 79.—The numbered areas show these four conditions:
1. An extraction from a penicillium culture, diluted 1:10, kills all bacteria in a circle 14 mm. in diameter.
2. Crude Penicillin, not diluted, in this Penicylinder affected a 20 mm. circle.
3. Crude Penicillin, highly concentrated then diluted 1:2,000, affected a 22 mm. area.
4. Extract in this Penicylinder has not inhibited any bacterial growth. (*The Laboratory*, **14**, No. 3, Fisher Sci. Co.)

(boils, carbuncles, abscesses, osteomyelitis, wounds, infections, etc.); infections due to *hemolytic streptococci* (skin infections, mastoiditis, peritonitis, puerperal sepsis—infection associated with child-bearing—etc.); pneumonia; gonorrhea; syphilis; etc.

However, penicillin has no effect in mitigating the evils of tuberculosis, malaria, typhoid fever, infantile paralysis, measles, mumps, influenza, and the like.

Blake, in comparing the action of penicillin with sulfonamides, devides the results into three groups:

1. Those in which both the sulfonamides and penicillin are more or less effective, though not necessarily equally so, namely certain Gram positive and Gram negative coccic infections: hemolytic streptococcus, pneumococcus, staphylococcus, Streptococcus viridans, meningococcus and gonococcus.

2. Those in which the sulfonamides are of value but not penicillin, namely Gram negative bacillary infections such as those caused by the

* See appendix p. 570.

colon bacillus, dysentery bacilli, Hemophilus influenzae, Friedländer's bacillus and Ducrey's bacillus.

3. Those in which penicillin is of value but not the sulfonamides, namely syphilis, yaws and possibly other spirochetal infections and those due to the Clostridia—gas gangrene.

How Does Penicillin Function?—In many ways penicillin acts much as do the sulfonamides—as bacteriostatic agents, preventing the growth of the organisms. However, there is evidence to show that, to some extent at least, penicillin also shows bacteriocidal properties—it kills the germs. This has a great advantage, in that the destruction of what remains of the infectious agent is not left entirely to the phagocytes, etc., of the body (as is the case with the sulfonamides), which may or may not be able to cope with the situation.

How Administered.—The *New and Nonofficial Remedies* of the Council of Pharmacy and Chemistry (*J. Am. Med. Assoc.*, 126, 367 (1944) states:

> Penicillin may be administered intravenously, intramuscularly, and locally. Subcutaneous injections may be painful. Treatment may consist of repeated intramuscular or constant intravenous injections. The contents of an ampul, or ampuls, are dissolved in sterile, pyrogen-free distilled water or isotonic solution of sodium chloride. For intravenous injection, concentrations of 1,000 to 5,000 units per cubic centimeter are prepared for direct injection, or 25 to 50 units per cubic centimeter for constant intravenous therapy; for intramuscular injection, 5,000 units per cubic centimeter of isotonic saline solution; for topical application (not the sodium salt in powder form, as it may be irritating when applied locally), 250 units, or more if infection is severe, per cubic centimeter of isotonic saline solution . . .

Notatin (also known as *penicillin B*).—Besides penicillin, *penicillium notatum* also manufactures a substance to which the name *notatin* has been given by some and which turns out to be an enzyme belonging to the flavoproteins (p. 154). This enzyme, which has been isolated, catalyzes the oxidation of glucose to gluconic acid and produces hydrogen peroxide:

$$C_5H_{11}O_5\,CHO + H_2O + O_2 \rightarrow C_5H_{11}O_5.COOH + H_2O_2$$

The anti-bacterial activity of notatin *in vitro* is due to the production of this hydrogen peroxide.

Tyrothricin.—Dubos isolated from the soil a sporulating bacillus which produces a soluble principle toxic for Gram-positive bacteria. Using peptone cultures of the soil bacillus, several fractions were obtained, each fraction characterized by showing marked bactericidal properties for Gram-positive bacteria.

Out of one such fraction a crystalline substance was isolated, and this was given the name *gramicidin*. It is soluble in acetone and alcohol, insoluble in water and ether and has a molecular weight of about 1400. It consists mainly of amino acids, probably in the form of polypeptides.

A substance somewhat similar to gramicidin, *tyrocidin*, has also been isolated.

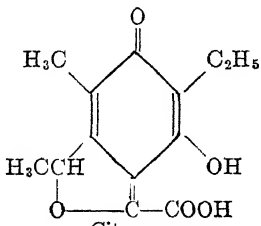
A dose of 0.005 mg. of gramicidin will kill *in vitro* 10^9 pneumococci

or group A streptococci in two hours at 30° C. One dose of 0.002 mg. of the substance injected into a mouse protects such an animal against 10,000 fatal doses of pneumococci or hemolytic streptococcus.

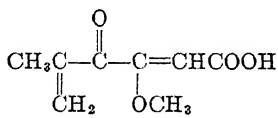
The mixture of gramicidin and tyrocidin—and possibly other substances—is known as *tyrothricin;* and while it is highly toxic when injected—and so cannot be considered in the same light as penicillin—it has come to be used locally in restricted cases; for example, superficial indolent ulcers, empyema (accumulation of pus in the chest or some cavity of the body); mastoiditis; and several wound infections.

Fumigatin, etc. Raistrick, Waksman and others have extracted various antibiotics from molds and, in some cases, have even isolated and determined the chemical structures of such substances. Because of their toxicity, none of them are used clinically

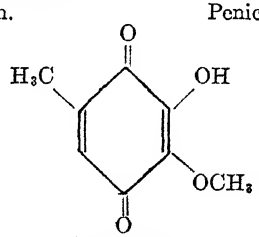
The probable structures of some of these substances are the following:



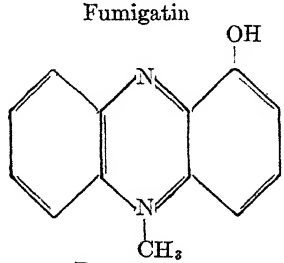
Citrinin.



Penicillic acid.



Fumigatin



Pyocyanine.

Streptomycin.—Penicillin has little effect on Gram-negative bacteria. Among the latter are the colon bacilli, organisms of the dysentery and typhoid group, others causing undulant fever, etc. The bacillus of tuberculosis, it is true, is Gram-positive, but penicillin has no effect.

A promising new antibiotic is streptomycin, isolated by Waksman from a medium growing *Actinomyces griseus.* Preliminary reports suggest that streptomycin may prove of value in tuberculosis, typhoid fever, undulant fever, etc.

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* Fumicacin is distinct from the pigment fumigatin, also produced by the same organism.

CHAPTER 15

CHEMISTRY OF RESPIRATION

THE process of respiration involves the absorption of oxygen through the lungs, the transfer of this oxygen by the blood to the cells, and the uptake of CO_2 by the blood, with its ultimate elimination through the lungs. What causes the oxygen to be taken up by the blood, and in quantity far beyond what can be explained on the grounds of a mere simple solution of the gas? What causes the oxygen to leave the blood and enter the cells? How is the elimination of carbon dioxide brought about? How, with the production of so much acid (not only carbonic but sulfuric and phosphoric acids from the oxidation of the sulfur and phosphorus in proteins) is the *p*H of the blood maintained in the neighborhood of 7.4?

This chapter is an attempt to answer these questions. Closely related problems will be taken up in subsequent chapters. For example, what is the mechanism involving oxidations within the cell (Chap. 19)? What are the energy relations involved in the process (Chap. 20)? What are the intermediate products formed when complex substances are oxidized (Chaps. 16, 17, 18)?

The air we breathe consists, approximately (in per cent), of oxygen, 20.96; carbon dioxide, 0.04; nitrogen, 79. The air we expire may have the composition: oxygen, 16.02; carbon dioxide, 4.38; and nitrogen 79. The essential reaction involves a consumption of oxygen and an elimination of carbon dioxide. The nitrogen as such is not utilized. It is true that cellular material contains nitrogen; but it is also true that the needs of the body for this element can be supplied only in the form of certain compounds of nitrogen (proteins, amino acids, lipids, etc.).*

When we come to the composition of the gases in the blood, we find that for human blood the figures are, approximately, as follows:

	Oxygen.	*Carbon dioxide.*	*Nitrogen.*
Arterial blood	19.45	49.68	1.7
Venous blood	14.04	54.65	1.7

These figures are in volumes per cent—the number of cc. of gas in 100 cc. of blood.

The Oxygen in the Blood.—A small quantity of oxygen is dissolved as such in the blood, but much the larger part is in combination with hemoglobin:

$$Hb + O_2 \rightleftarrows HbO_2$$

It is this property of hemoglobin to take up oxygen at one pressure and

* Man can continue to exist for weeks without food and for days without water; but without oxygen he dies within a few minutes.

to give it up at a lower pressure which is at the very basis of one of life's activities.

At standard conditions pure oxygen will dissolve in water to the extent of about 2.2 cc. of gas in 100 cc. of liquid (2.2 volumes per cent). If air be used in the place of oxygen, then the amount of oxygen dissolved would be (roughly) 0.4 volumes per cent. This is based on the well-known principle that, in a mixture of gases, the volume of a gas dissolved will depend upon its partial pressure. If the pressure of air is 760 mm. of Hg, then the partial pressure due to oxygen is 150 mm. of Hg. But actually the partial pressure of oxygen in arterial blood is nearer 80 mm. of Hg. Hence, if the oxygen were present in blood merely in the form of a dissolved gas, the amount would be about 0.2 volumes per cent. Actually, the amount of oxygen in blood is about 100 times as great.

Baldwin gives the oxygen capacities of some different bloods as follows (Table 43):

TABLE 43.—OXYGEN CAPACITIES OF SOME DIFFERENT BLOODS. (Baldwin, *Comparative Biochemistry*, Cambridge Univ. Press, London.)

Pigment.	Color.	Site.	Animal.	Cubic centimeters oxygen per 100 cc. blood.
Hemoglobin	Red	Corpuscles	Mammals	25
			Birds	18.5
			Reptiles	9
			Amphibia	12
			Fishes	9
		Plasma	Annelids	6.5
			Molluscs	1.5
Hemocyanin	Blue	Plasma	Molluscs:	
			Gastropods	2
			Cephalopods	8
			Crustaceans	3
Chlorocruorin	Green	Plasma	Annelids	9
Hemerythrin	Red	Corpuscles	Annelids	2

Either by removing hemoglobin from blood, or by determining its oxygen-combining capacity, or by determining the amount of oxygen evolved from hemoglobin when the pressure is reduced (*in vacuo*), it can be shown that by far the larger amount of oxygen in the blood is in combination with hemoglobin.

How Do the Tissues Get Oxygen?—The tissues get oxygen as a result of a drop in partial pressure which releases some of the gas from its combination with hemoglobin.

Let us examine the oxyhemoglobin dissociation curves (Fig. 80). When the partial pressure of oxygen is 150 mm. of Hg (its partial pressure in air), hemoglobin will combine with oxygen to the extent of 20 volumes per cent (that is, 20 cc. of oxygen in 100 cc. of blood).

This represents the saturation point; because increasing the partial pressure of oxygen 150 mm. of Hg causes no further combination of the gas with the pigment.

When, however, the pressure is lowered below 150 mm., then the oxyhemoglobin begins to dissociate and oxygen is set free. At 80 mm. pressure—which is approximately the pressure in arterial blood—but 19 cc. of oxygen is dissolved in 100 cc. of blood (19 volumes per cent), and at this stage hemoglobin is saturated with the gas to the extent of 95 per cent (19/20 of 100).

The pressure of oxygen in venous blood is 35 mm. of Hg. The blood is saturated to the extent of 60 per cent, and it therefore has 12 volumes per cent of oxygen (see Fig. 80). The lowering of the partial pressure means that oxygen has passed from the blood to the tissues.

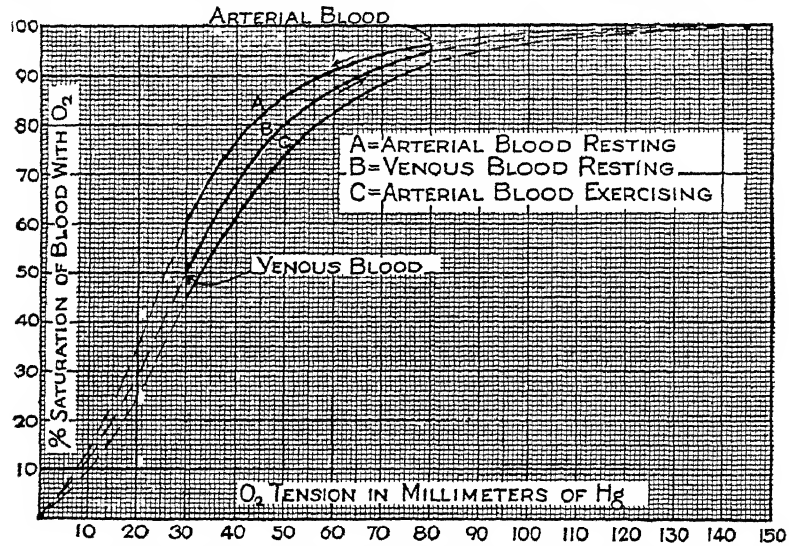


Fig. 80.—Oxyhemoglobin dissociation curves indicating changes occurring in venous blood during rest and arterial blood in exercise. (Himwich.)

In the tissue fluid surrounding the capillaries, the partial pressure of oxygen may be between 20 and 40 mm. It is interesting to note that within this critical range, a lowering of pressure by 20 mm., say, will cause a larger release of oxygen than a corresponding drop at higher pressures. For example, at 40 mm., the blood is 60 per cent saturated; at 20 mm. it is 30 per cent saturated. This means that a lowering of the pressure from 40 to 20 mm. liberates 8 volumes per cent of oxygen (40/100 × 20). On the other hand, at 90 mm. the percentage saturation is 95, and at 70 mm. it has scarcely dropped to 94; liberating, therefore, about 0.5 volumes per cent of oxygen.

The affinity of oxygen for hemoglobin is dependent not only upon the partial pressure of the oxygen but also upon the *p*H of the solution. In a general way, it may be said that, within certain limits, the greater the acidity, the greater the tendency for oxyhemoglobin to dissociate. This is important, for example, during exercise, when the blood tends

to become less alkaline. Here we find (Fig. 80) that the arterial blood at 40 mm. pressure is almost 80 per cent saturated in the absence of violent exercise, and about 60 per cent saturated as a result of such exercise. This means that, during such a period of stress, more oxygen will be available for the tissues. The carbon dioxide which is liberated from the tissues has, of course, the tendency to increase the acidity of the medium and, therefore, to increase the dissociation of hemoglobin.

The Carbon Dioxide in the Blood.—One hundred cc. of blood contains from 40 to 60 cc. of carbon dioxide—nearer 40 cc. if arterial blood and close to 60 cc. if venous blood. This is determined by withdrawing blood under carefully controlled conditions, treating it with an excess of a weak acid (lactic acid, for example), and measuring the amount of CO_2 evolved *in vacuo*. From 2 to 2.5 cc. of the gas is in simple solution. In what state is the rest? And how is the CO_2 eliminated by the lungs?

In the first place the partial pressure of CO_2 in the arterial blood is some 35 mm., and in the tissues it is increased to 50 to 70 mm., due to its production as a result of metabolic activity. The net result is a tendency for CO_2 to pass from the tissues into the blood.

But the problem involving carbon dioxide is much more complicated. Little is carried by the blood as carbon dioxide itself. Furthermore, the production of carbonic acid might well lead to diminished alkalinity, which, in turn, would produce abnormal results in the body. Yet, as a matter of fact, despite such a possibility, the *p*H of the blood varies surprisingly little.

Van Slyke states the problem in the following very graphic form: we know that the arterial blood in man contains about 50 volumes per cent of carbon dioxide, and the venous blood about 55 to 60. In a pure solution this quantity of carbon dioxide would give a hydrogen ion concentration of 3.1×10^{-5}. The hydrogen ion concentration of blood is about 1/1000 as great.

The answer to this problem is found in several factors which come into play. One of these factors is the buffers of the blood—the proteins and bicarbonate of the plasma, and the proteins, phosphates, and bicarbonates of the cells. Another factor depends upon the acidic properties of oxyhemoglobin and hemoglobin: the former is a stronger acid and prevents the plasma from becoming more alkaline in the lungs and more acid in the tissues. Still another factor is what is known as the "chloride shift," whereby bicarbonate ion flows from the red cells into the plasma in exchange for chloride ion—a further contribution toward "neutrality" conditions. The more recent developments deal with still another phase of the carbon dioxide problem—the rapidity with which the gas is removed from the lungs.

These various factors will be discussed in turn.

Forms in Which CO_2 Exists in Blood.—The carbon dioxide exists largely as carbonic acid and as sodium bicarbonate. There may be some dissolved CO_2. Considering the amount of carbonic acid in the blood, the likelihood of any sodium carbonate being present may be dismissed. Using 0.155 per cent of sodium carbonate (equivalent to

the concentration of carbonate in plasma), and in the presence of carbon dioxide at a pressure of 45 mm. (corresponding approximately to the pressure of CO_2 in the blood), Bohr showed that 99.5 per cent of the CO_2 is in the form of bicarbonate.

Much depends, then, upon the proper balance between the carbonic acid and the sodium bicarbonate, if the alkalinity of the blood is to be kept within physiological limits. "If all the CO_2 of the blood," writes Van Slyke, "were in the free form as H_2CO_3, the blood would be 1000 times more acid than it is. . . . If all the CO_2 were in the form of carbonates . . . the blood would be hundreds of times too alkaline." If we remember that the extremes of life range between a *p*H of blood of 7 to 7.8 then the importance of having an adequate buffering system becomes apparent (Fig. 81).

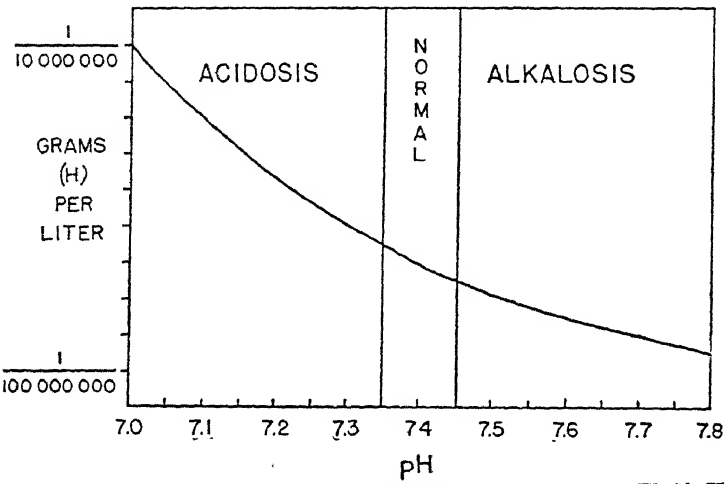


Fig. 81.—Possible Range of *p*H in Blood. (Gamble, *Extracellular Fluid*, Harvard Med. School).

The Buffers of the Blood (reread pp. 556–558).—We have had occasion to refer to buffers and their action. The subject in connection with blood is so important, that it must be discussed again.

The buffers of the blood are

$$\frac{H_2CO_3}{BHCO_3'}\ \frac{BH_2PO_4}{B_2HPO_4'}\ \frac{HHbO_2}{BHbO_2'}\ \frac{HHb}{BHb'}\ \frac{HPr}{BPr}$$

(where B = base, Hb = hemoglobin and Pr = protein).

The plasma contains most of the bicarbonate and albumin and globulin, whereas the corpuscles contain the hemoglobin and most of the phosphates.

We have already seen (p. 551) that

$$[H] = K\frac{[Acid]}{[Salt]}$$

To indicate the degree of dissociation of the salt (which in 0.1 to 0.01 molar strength is from 60 to 90 per cent), we shall use the ex-

pression λ; so that, more accurately

$$[H] = K\frac{[Acid]}{\lambda[Salt]}$$

If we take into account the range of possible concentrations within blood itself, λ remains practically constant; so that we can say that $K/\lambda = K_1$, a new constant; or

$$[H] = K_1\frac{[Acid]}{[Salt]}$$

Converting the equation into the *p*H form, and remembering that *p*H is the negative logarithm of $[H^+]$

$$pH = -\log K_1 - \log\frac{[Acid]}{[Salt]}$$

or,

$$pH = pK_1 + \log\frac{[Salt]}{[Acid]}$$

where $pK_1 = -\log K_1$.

Where the salts in question are phosphates—where, in other words, the salts are $\frac{B_2HPO_4}{BH_2PO_4}$, $pK_1 = 6.8$. Where the salts are the carbonates $\left(\frac{BHCO_3}{H_2CO_3}\right)$, $pK_1 = 6.1$.

It is obvious, then, that knowing the pK_1 and the salt concentration, the *p*H can be determined.

It is seen, too, that the hydrogen ion concentration of the buffer solution is proportional to the ratio $\frac{[Acid]}{[Salt]}$; and from what has already been said (pp. 557–558) when the ratio $\frac{HA}{BA} = 1$, and $[H] = K_1$, the curve changes least, giving us maximum buffer effects (Fig. 82). In other words, when we have 50 per cent of the CO_2 as $BHCO_3$, the addition of either acid or alkali causes less change in *p*H than at any other point on the curve.

With blood at *p*H 7.4, the $\frac{BHCO_3}{H_2CO_3} = \frac{20}{1}$. Where $\frac{BHCO_3}{H_2CO_3} = 1$, *p*H = 6.1.* With the phosphate, when $\frac{B_2HPO_4}{BH_2PO_4} = 1$, *p*H = 6.8.

If, for any reason, the *p*H of the blood is lowered (as in acidosis), then these buffers become much more efficient.

In so far as the oxyhemoglobin is concerned, its maximum buffering effect is at *p*H 7.2, which is appreciably nearer to the normal *p*H of blood.

* The control of carbon dioxide by the respiratory system makes the carbonic acid-bicarbonate buffering in blood mueh more effective than *in vitro*.

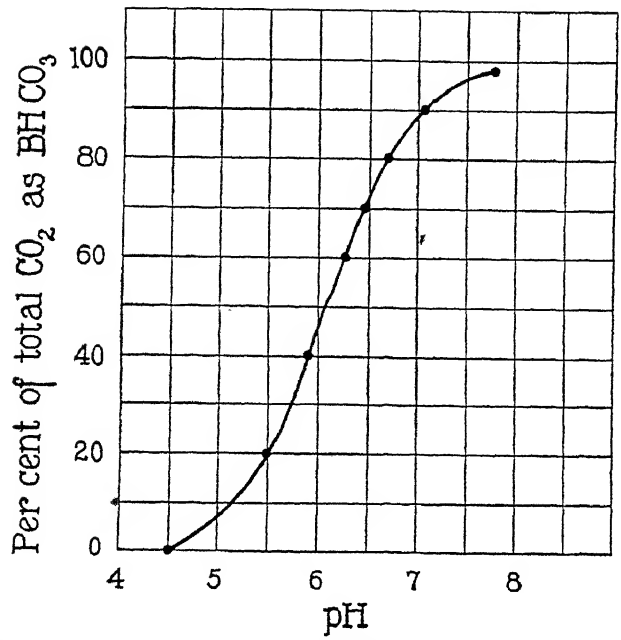


Fig 82.—Action of $NaHCO_3$:H_2CO_3 buffer, showing maximum buffer effect at middle of curve when $NaHCO_3$:H_2CO_3 ratio = 1. (Van Slyke, *Physiol Rev*, 1, 147)

The Distribution of Buffering Capacities.—That cells are richer in buffering capacity than the plasma has been known for a long time. By determining the CO_2 absorption curves of whole blood and of separated serum, it can be shown that the increase in combined CO_2 in whole blood is more than three times that in serum. The cell buffers are hemoglobin and phosphate, neither of which diffuses into the plasma. Despite this fact, these buffers exert their influence on the plasma. The estimated distribution, which can be given only very approximately, is seen in Table 44.

TABLE 44.—ESTIMATED APPROXIMATE DISTRIBUTION OF CO_2 CARRYING POWER AMONG BUFFERS OF OXYGENATED BLOOD. (Van Slyke)*

Buffer.	CO_2 carrying power of separate blood buffers between *p*H 7.35 and 7.25.	Proportion of CO_2 carrying power of the blood.
	vol per cent	*per cent*
Bicarbonate	0.5	6
Plasma protein	1.0	13
Cell phosphate	2.0	25
Oxyhemoglobin	5.8	72
Total by addition	9.3	116

* This table is not strictly true. As Greenwald points out, it refers to the effect of acid added to blood *without the removal of carbon dioxide*—which is not a true picture of buffers operating in the organism.

Hemoglobin-Oxyhemoglobin.—Aside from its buffering properties, the change which hemoglobin undergoes when it is converted into oxyhemoglobin (and vice versa) has an important bearing on the passage and ultimate elimination of the CO_2. At the lungs the hemoglobin combines with the oxygen to form oxyhemoglobin. In the tissue capillaries, with a lowered oxygen tension, oxygen passes from the blood into the cells. In the meantime, differences in partial pressure of the CO_2 in blood and tissues cause this gas to leave the tissues and enter the blood.

Oxyhemoglobin is a stronger acid than hemoglobin. pK for the former is 6.62 and for the latter, 8 18. Both these substances are present as salts and as free acids. In the tissues the change from the oxyhemoglobin to hemoglobin is also accompanied by a release of base which neutralizes some of the carbon dioxide. In the lungs, with the change of hemoglobin to oxyhemoglobin, the latter acts on the bicarbonate to liberate carbon dioxide.

The Chloride Shift.—Chlorides are found in the various fluids and cells of the body. Apparently, the cell membrane does not inhibit the passage of chloride ions. Yet the concentration of this inorganic ion in the blood cells is about half that in the blood plasma. This is due, according to Van Slyke, to two causes: (1) in the cells nearly half of the base, which is potassium, is combined with the hemoglobin, so that approximately one-half of the base is available for combination with chloride and bicarbonate; in the plasma but one-tenth of the base, which is sodium, is combined with protein; so that relatively more base is available for combination with the chloride and the bicarbonate; (2) the cells contain 65 per cent of water, whereas the plasma contains 92 per cent. The electrolyte concentration per cubic centimeter of cells is less than that per cubic centimeter of plasma, for per gram of water "the total concentration of osmotically active electrolytes is equal in cells and plasma."

As the carbon dioxide tension of the blood is changed, there is a change in the distribution of chloride and bicarbonate between cells and plasma. An increase in CO_2 increases H_2CO_3. The acid combines with the base which had hitherto been attached to the hemoglobin:

$$H_2CO_3 + K_2Hb \rightleftarrows KHCO_3 + KHHb$$

The equilibrium is upset by the additional formation of bicarbonate. To restore this equilibrium, some bicarbonate ion passes from the cells to the plasma, and an equivalent amount of chloride ion leaves the plasma for the cells.

The results may be shown graphically as follows:

(1) CO_2 enters the blood (cells and plasma):

Plasma.	*Cells.*
CO_2	CO_2
$CO_2 + H_2O \rightarrow H_2CO_3$	$CO_2 + H_2O \rightarrow H_2CO_3$
NaCl	K_2Hb
$NaHCO_3$	KCl
	$KHCO_3$

(2) Reaction of H_2CO_3 with K_2Hb:

Plasma.	*Cells.*
NaCl	$K_2Hb + H_2CO_3 \rightarrow KHHb + KHCO_3$
$NaHCO_3$	KCl
H_2CO_3	H_2CO_3

(3) Redistribution:

Plasma.	*Cells.*
Na (Cl) →	
	K (HCO_3)
←	
$NaHCO_3$	KHHb
H_2CO_3	H_2CO_3
	KCl

A Summary of CO_2 Transport.—Before taking up the next advance—the discovery of carbonic anhydrase—a summary of the discussion so far may help to clear the situation. The CO_2 forms carbonic acid and alkali bicarbonate. At the *p*H of the blood, little carbonic acid as such is present. Of the buffers supplying the bicarbonate with alkali, the most important is hemoglobin. Furthermore, when CO_2 appears in the blood, the oxyhemoglobin loses its oxygen, and the base released in this transformation becomes available for more bicarbonate. In the meantime, owing to a concentration of buffer salts in the corpuscle, the HCO_3^- tends to drift in that direction. To retain equilibrium, there is an interchange of chloride and bicarbonate ion (as has just been explained).

Carbonic Anhydrase.—With the discovery of an enzyme which accelerates the decomposition of carbonic acid,* a certain modification in point of view has become necessary.

In 1928 Henriques showed that the rate of escape of CO_2 from serum was less than that from hemoglobin. This suggested the possibility that the red cells contain a catalyst. Even when diluted 1:20,000, the hemoglobin still retained the accelerating effect in decomposing bicarbonate (in presence of buffers at *p*H 7.4). Heme itself was devoid of catalytic properties. On the other hand, globin acted as efficiently as hemoglobin itself (Roughton). However, the catalyst proved to be neither hemoglobin nor globin, but a substance associated with them.

The enzyme can be separated from the red cells by adding water and alcohol, and then chloroform (to coagulate the hemoglobin). The mixture is centrifuged. The top layer of solution contains the catalyst. By evaporating to dryness in a vacuum desiccator, the impure carbonic anhydrase is obtained in stable form.

The enzyme cannot be dialyzed and is destroyed by heating for thirty minutes at 65° C. It is stable over a *p*H range of 4 to 12, and the purest material so far obtained shows the usual protein tests. The enzyme is absent in plasma and seems to be entirely concentrated in the red cells.

The enzyme has been isolated. It is a protein containing zinc as an essential element.

* Under certain conditions, the enzyme can influence the hydration of CO_2 as well as the dehydration of H_2CO_3.

Carbamino Compounds.—Further work on the rate of evolution of CO_2 suggested to Henriques and to Roughton that some of the CO_2 is very possibly combined with protein in the form of a carbamino compound (carbhemoglobin):

$$CO_2 + PrNH_2 \rightleftarrows PrNHCOOH \rightleftarrows PrNHCOO^- + H^+$$

The various forms, then, in which CO_2 exists in blood are given by Roughton as follows (Fig. 83):

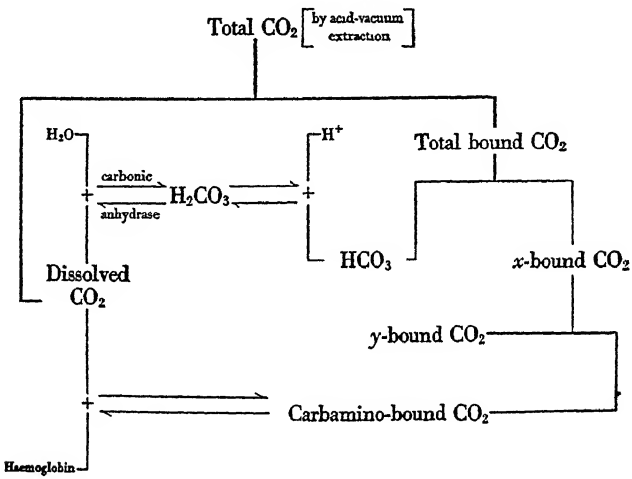


Fig. 83.—Various forms in which carbon dioxide is believed to be present or combined in blood, and their interrelationships. [Roughton, *Physiol. Rev.*, **15**, 243.]

Of the total bound CO_2, part is in the form of bicarbonate, and of the remainder (x-bound CO_2), some is in the carbamino form, and some in some other, unknown form (y-bound CO_2).

A summary of respiratory exchanges between cells and plasma is given in Fig. 84.

The Kidney and Body Neutrality.—So far we have discussed in large part the manner in which the body handles carbonic acid. We have seen how the buffers of the blood tend to minimize the effect of this acid, and we have also seen how the lungs, by eliminating CO_2, tend to prevent its accumulation. However, when food products are burnt in the cells, acids other than carbonic are also produced. For example, protein material and protein decomposition products contain such elements as sulfur and phosphorus, and these are oxidized to sulfuric and phosphoric acids, respectively. In the blood these acids are neutralized, and then their salts are eliminated by the kidney.

As the *p*H of the urine is usually slightly on the acid side, the strong acids can only be eliminated in the form of their salts. Whereas there is a preponderance of Na_2HPO_4 over NaH_2PO_4 in the blood, the reverse is true in the urine.

Where, as a result of the formation of salts of sulfuric, phosphoric, and hydrochloric acids (and often organic acids), there is danger of a

too extensive withdrawal of fixed bases from the blood, with a consequent production of an acidosis, the fixed bases are replaced by ammonia, which is largely produced for the occasion by the kidney. Normally, the deaminized portion of an amino acid is largely converted into urea; but where there is danger of an excessive loss of fixed alkali, some of the ammonia serves the purpose of neutralizing some of the acid.

Where, for some reason, the body produces too much alkali rather than too much acid, the excess, in the form of Na_2HPO_4 and $NaHCO_3$, is eliminated through the kidney.

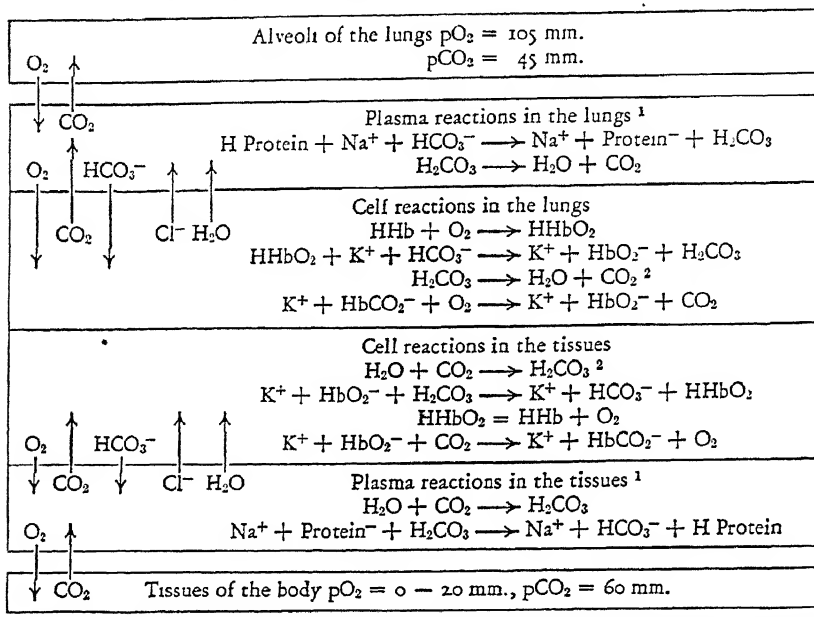


Fig 84.—Diagrammatic representation of the distribution of blood constituents which play an important role in respiratory exchanges between cells and plasma, in arterial and venous blood, at rest and at work. (Adapted from Johlin, *Physical Biochemistry.*)

The salts of organic acids (such as acid potassium citrate), found in certain foods, are oxidized in the body to carbonates, and the CO_2 is eliminated in the usual way through the lungs. During violent exercise, with an overproduction of lactic acid, this substance will be excreted via the kidney, partly as a salt of a fixed base, partly as an ammonium salt, and partly as the acid itself.

In abnormal conditions, as in diabetes, with the production of acetone bodies (β-hydroxybutyric acid, acetoacetic acid, and acetone), the two acids are eliminated in the urine largely in the form of salts, though in advanced stages of such an acidosis, the free acids themselves may appear. An added protective mechanism at this stage is the conversion of some of the acetoacetic acid into acetone, a neutral substance eliminated through the kidneys and the breath, and CO_2, eliminated through the lungs.

TABLE 45.—DISTURBANCES OF ACID-BASE EQUILIBRIUM OF BLOOD. [Hawk and Bergeim, *Practical Physiological Chemistry* (1937), p. 497, The Blakiston Co., publishers.]

Area	Acid-base balance.	Conditions.	Associated symptoms.	Compensatory mechanisms.
1 Uncompensated alkali excess	[$BHCO_3$] increased without proportionate rise in [H_2CO_3], therefore *p*H increased.	Overdosage of $NaHCO_3$ Excessive vomiting (pyloric obstruction) or gastric lavage (loss of HCl) *x*-Ray or radium treatment	If marked, tetany.	Diminished respiration (rise in alveolar CO_2) to hold back CO_2 Diuresis and increased $NaHCO_3$ excretion
2–3 Uncompensated CO_2 deficit	[H_2CO_3] decreased without proportionate fall in [$BHCO_3$], therefore *p*H increased.	Hyperpnea, voluntary or induced (oxygen want, *e. g.*, at high altitudes) Fever. Hot baths.	If marked, tetany	Retention of acid metabolites (low NH_3 and titratable acidity of urine) Excretion of $NaHCO_3$.
4 Compensated alkali or CO_2 excess	[$BHCO_3$] (or [H_2CO_3]) increased but balanced by proportionate rise in [H_2CO_3] (or [$BHCO_3$]), therefore *p*H normal	*Alkali excess* $NaHCO_3$ therapy, with slow absorption. *CO_2 excess.* Retarded gas exchange (*e. g.*, emphysema) with CO_2 tension chronically increased.	Cyanosis due to deficient oxygen exchange.	CO_2 retention. $BHCO_3$ retention.
5 Normal.	[$BHCO_3$] and [H_2CO_3] normal at ordinary altitudes.			
6 Compensated alkali or CO_2 deficit.	[$BHCO_3$] (or [H_2CO_3]) decreased but balanced by proportionate fall in [H_2CO_3] (or [$BHCO_3$]), therefore *p*H normal.	*Alkali deficit.* Accelerated production (*e. g.*, diabetes) or retarded elimination (*e. g.*, nephritis) of nonvolatile acids. Experimental acid intoxication. Diarrheal acidosis of infancy (marasmus). *CO_2 deficit.* Overventilation at high altitudes (oxygen want).	Hyperpnea.	Increased respiration ("blowing off CO_2"). Accelerated NH_3 formation and acid excretion. Same as in Areas 2 and 3.
7–8 Uncompensated CO_2 excess	[H_2CO_3] increased without proportionate rise in [$BHCO_3$], therefore *p*H decreased.	Retarded respiration as in pneumonia (physical obstruction) or morphine narcosis (deadening of respiratory center). Experimental rebreathing. Cardiac decompensation.	Dyspnea.	Increased respiration. Accelerated NH_3 formation and acid excretion. Probable shift of acid from blood to tissue
9 Uncompensated alkali deficit.	[$BHCO_3$] decreased without proportionate fall in [H_2CO_3], therefore *p*H decreased.	Terminal stages of nephritic acidosis, and diabetic acidosis (compensated by insulin therapy). Deep ether anesthesia. Certain cardiac cases. Eclampsia.	Dyspnea.	Increased respiration. Increased acid excretion and NH_3 formation (except probably in nephritis).

Acidosis and Alkalosis.—According to Peters and Van Slyke, acidosis is a condition resulting from an abnormal accumulation of acid, or from an abnormal loss of alkali. This may mean a lowering of the *p*H and a decrease in the amount of bicarbonate. Alkalosis, on the other hand, results from an abnormal accumulation of alkali, or from an abnormal loss of acid. This means an increase in the amount of bicarbonate and, as a rule, an increase in the *p*H. Where the abnormality is due to carbonic acid—where there may be too little or too much of this acid—the amount of bicarbonate and the *p*H do not necessarily follow one another in the same direction.

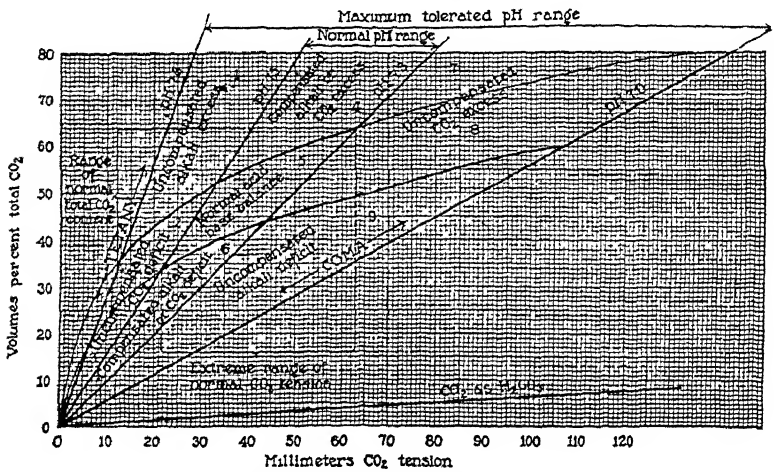


Fig. 85.—Normal and abnormal variations of the $BHCO_3$, H_2CO_3, CO_2 tension and *p*H in oxygenated human whole blood drawn from resting subjects at sea level. (Van Slyke)

In diabetes, in terminal stages of chronic nephritis, in shock, burns, poisoning, and fasting, a severe acidosis sets in which may be of clinical significance. The common form of acidosis, seen in diabetes, is due to an abnormal production of β-hydroxybutyric acid, $CH_3.CHOH.CH_2COOH$, and acetoacetic acid, $CH_3.CO.CH_2.COOH$.

Where alkalis are administered in such amounts that their elimination by the kidney is retarded and they accumulate in the blood, we get the abnormal increase in the alkali reserve of the body (alkalosis). The same condition may arise from overventilation, with its excessive breathing, leading to a loss of carbon dioxide and an increase in the *p*H of the blood.

The disturbances in the acid-base balance may be classified as shown in Table 45 and Fig. 85.

Experimental Methods.—The methods used for determining the acid-base balance of the body may be found in standard texts (see references at the end of the chapter). Here the principle of but one of several methods will be discussed. In this particular case, we measure the alkali reserve of the body by determining the CO_2 capacity of the plasma. Plasma saturated with CO_2 is acidified, and the CO_2 is liberated

in a partial vacuum. The volume of CO_2, measured at standard conditions, is calculated on the basis of 100 cc. of plasma, giving the results in volumes per cent (x cc. of CO_2 in 100 cc. of plasma).

A decrease in the alkali reserve of the body will mean that less CO_2 can exist in combination with alkali, and that, therefore, less CO_2 is eliminated after treatment with acid. The normal value varies from 80 to 53 volumes per cent of CO_2. In mild acidosis, it is lowered to 53 to 40; in moderate to severe acidosis, to 40 to 30; and in severe acidosis, to below 30.

References

An excellent introduction to the subject may be found in Johlin, *Introduction to Physical Biochemistry* (1941), chapters 2, 7, 10 and 11. See also, Cantarow and Trumper, *Clinical Biochemistry* (1945), chap. 13.

Material pertaining to this chapter will be found in Gamble's *Extracellular Fluid* (Harvard Medical School, 1942)—a splendid presentation; Bull's *Physical Biochemistry* (1943); and West's Physical Chemistry (1942).

The general article by Himwich in Harrow and Sherwin's *Textbook of Biochemistry* (1935), p. 462, is strongly recommended.

In connection with carbonic acid and the acid-base balance, see Peters and Van Slyke's *Quantitative Clinical Chemistry* (1932), vol. 1, p. 868. See, also, the articles by Van Slyke, *Physiol. Rev.*, **1**, 141 (1921), Wilson, *Physiol. Rev.*, **3**, 295 (1923); and Sendroy, *Ann. Rev. Biochem.*, **7**, 231 (1938).

The work on the CO_2 transport by the blood is reviewed in masterly fashion by Roughton, *Harvey Lectures* (1943–44), p. 96.

A fascinating book, by a pioneer in this field, is Henderson's *Blood: A Study in General Physiology* (1928).

Individual papers, to give the reader a "taste" of some of the experimental methods used, are the following: Henderson, *J. Biol. Chem.*, **41**, 401 (1920) (equilibrium between O_2 and CO_2 in blood); Van Slyke, Wu, and McLean, *J. Biol. Chem.*, **56**, 765 (1923) (electrolyte and water distribution in blood); and Stadie and O'Brien, *J. Biol. Chem.*, **117**, 439 (1937) (carbamate equilibrium).

For the work on carbonic anhydrase, see Meldrum and Roughton, *J. Physiol.*, **80**, 113 (1933); Keilin and Mann, *Biochem. J.*, **34**, 1163 (1940) (zinc is present in the enzyme); Scott and Mendive, *J. Biol. Chem.*, **140**, 445 (1941) (the enzyme is a protein); Scott, *Ibid.*, **142**, 959 (1942) (a crystalline preparation); Kiese and Hastings, *Ibid.*, **132**, 281 (1940), and Main and Locke, *Ibid.*, **140**, 909 (1941) (factors affecting activity); Hove, Elvehjem and Hart, *Ibid.*, **136**, 425 (1940) (relation of zinc to the enzyme); Mann and Lutwak-Mann, *Ann. Rev. Biochem.*, **13**, 41 (1944) (recent advances).

CHAPTER 16

METABOLISM OF CARBOHYDRATES

As a result of digestive processes, carbohydrates are largely in the state of hexoses when absorption begins. Our problem is to discuss what happens to carbohydrate from the point where it is absorbed to the point where it is eliminated in the form of carbon dioxide and water.

The field is a complex one. It deals with the storage of glycogen in liver and in muscle; with the glucose of the blood and the manner in which its amount is controlled; and with the oxidation of carbohydrate, involving a number of reagents and a number of intermediary products. These topics will be taken up in turn.

The Liver.—This organ is so intimately concerned with the mechanism of carbohydrate metabolism (and of fat and protein metabolism too, as we shall see later) that a few preliminary remarks are necessary.

Aside from the secretion of bile and its property of detoxicating toxic substances—to name but two properties of this organ—a third characteristic of the liver is what concerns us particularly in this chapter: its relation to the utilization of food. The liver stores, manufactures and regulates food materials. Not only are carbohydrates handled—as we shall see in the next paragraph—but so are fats (p. 334) and proteins (in the form of amino acids, p. 349).

Some vitamins, vitamin A, for example (p. 138), are stored to an appreciable degree in the liver; and so is the substance effective in pernicious anemia (p. 260).

Several syntheses occur in this organ. Heparin (p. 270), for example, seems to be manufactured here. So, probably are the plasma proteins (p. 263) and fibrinogen and prothrombin (p. 268)—the last apparently through the medium of vitamin K(p. 190).

Glycogen in Liver.—The carbohydrates, absorbed very largely in the form of hexoses, pass into the liver, where some are stored in the form of glycogen,* and the rest empty into the general circulation. When glycogen is hydrolyzed, glucose alone is obtained; and, normally, the one sugar in circulating blood is glucose. These and other changes in the body involve the action of enzymes (p. 89). It is still an unsettled problem whether the fructose and galactose—and this includes the less common mannose—are first transformed into glucose and then

* By injecting deuterium (in the form of D_2O) into rats kept on a high carbohydrate diet, Stettin was able to show an appreciable increase in isotopic concentration in the glycogen isolated from the liver and carcass. The liver fatty acids also showed a decided increase in isotopic concentration. In fact, on a high carbohydrate diet, more of the glucose (in the food) was used to synthesize fatty acids (fat for the body) than to form glycogen.

synthesized into glycogen, or whether each one of these sugars is directly synthesized into the polysaccharide.

It is also possible that such monosaccharides are broken down to C_3 compounds—possibly lactic acid—which can be polymerized into glycogen, and the latter, in turn, can then be hydrolyzed to glucose. In any case, the normal sugar of circulating blood is glucose, quite irrespective of the carbohydrate material at the time of absorption.

We may say at once that one of the important factors controlling the amount of glucose in the blood is the glycogen reserve in the liver. If the liver is removed from an animal there is a marked drop of glucose in the blood (a hypoglycemia), even though the muscles retain appreciable quantities of glycogen. In other words, a controlling factor in the amount of glucose in the blood is the conversion of liver glycogen into glucose. The blood sugar is not derived from the glycogen of muscle (except indirectly; see p. 314).

As a matter of fact, not only are the three common monosaccharides stored as glycogen in the liver—the amount stored depending upon the various requirements of the body—but a number of amino acids, and the glycerol portion of the molecule of fat, are also "glycogen formers" in various degrees, and thereby help to add, if necessary, to the ultimate glucose supply. Among amino acids, mention may be made of glutamic acid, cystine, alanine, proline, serine, and aspartic acid. They first undergo deamination, whereby the NH_2 group is ultimately converted into urea (p. 353), the rest of the molecule being polymerized into glycogen in a manner quite unknown to us.

While glycerol itself is a "glycogen former," there is little evidence that the fatty acids functions similarly.* Neither do pentoses have the ability to form glycogen. On the other hand, not only is glycerol of value in the formation of the polysaccharide in liver, but several other three-carbon compounds resemble it. For example, dihydroxyacetone, $CH_2OH.CO.CH_2.OH$, forms liver glycogen more readily than galactose, and is almost a rival of glucose and fructose. Lactic acid, $CH_3.CHOH.COOH$, methylglyoxal, $CH_3.CO.CHO$ (also called pyruvic aldehyde), and pyruvic acid, $CH_3.CO.COOH$, are also capable of being transformed into glycogen in the liver, though not so efficiently as dihydroxyacetone.

It is such experiments dealing with three-carbon compounds that led to views regarding intermediate carbohydrate metabolism. The reasoning was as follows: three-carbon compound A forms glycogen in the liver; the latter, when hydrolyzed, always yields glucose; from which we may, perhaps, conclude that when the tissues oxidize the glucose to carbon dioxide and water; the glucose molecule is first degraded to the three-carbon compound A. In this way, dihydroxyacetone, lactic acid, etc., have featured prominently as "intermediates" in carbohydrate metabolism.

It may be said that experiments on isolated livers, perfused with

* However, Hastings has shown that among *lower* fatty acids, such as propionic and butyric, a certain conversion to glycogen is possible. This, however is not true of acetic acid.

Ringer's solution containing three-carbon compounds, have led to similar results.

As showing how varied may be the source of the glycogen of the liver, we find very appreciable quantities of the latter on a diet devoid of carbohydrate altogether. As we have already indicated, the glycerol in fat, and a number of amino acids in protein, serve very well as "glycogen formers." So true is this, that even in starvation, which first depletes the stored carbohydrates, we still find some glycogen in the liver, derived from the breakdown of proteins and fats in the tissues, and also from lactic acid formed in muscle from glycogen (see below).

The Glucose of the Blood.—The glucose of the blood is remarkably constant under normal conditions. It rarely rises (in the "normal" human) much above 100 mg. per 100 cc. of blood (the range is about 80–120 mg.) The regulatory mechanism is controlled by a number of factors: the formation of glycogen in the liver; the formation of glycogen in muscle, and to a lesser extent, in other tissues; the oxidation of carbohydrate; the conversion of carbohydrate into fat; and the excretion of glucose. The hormones of the pancreas and the pituitary play a dominant role.

The Glycogen in Muscle.—The source of muscle glycogen is the glucose of the blood. As the glycogen of the muscle is used up—during exercise, for example—some of the glycogen in the liver is converted to glucose, which then passes into the blood, and serves as source material for the replenishment of the glycogen in muscle.

It can be shown that insulin, the hormone in the pancreas, is involved in the formation of glycogen in muscle. In a depancreatized dog, the injection of glucose alone does little to influence the amount of the polysaccharide in muscle; but when both glucose and insulin are given, the amount increases very perceptibly. Even in normal animals, the injection of insulin increases the glycogen deposit in the muscle. This is not true in so far as the liver is concerned. Only in the animal suffering from diabetes will the injection of insulin cause an increased deposition of glycogen in the liver.

The contraction of muscle forms lactic acid. Some of this acid finds its way into the blood, thence to the liver, where it is synthesized to glycogen (Cori). The relationship may be outlined thus:

(A) Liver glycogen → (B) Blood sugar → (C) Muscle glycogen → (D) Blood lactic acid → (A) Liver glycogen

Epinephrine (adrenaline), from the adrenal glands, accelerates the conversion of muscle glycogen to blood lactic acid and of liver glycogen to blood sugar; and insulin accelerates the change from blood sugar to muscle glycogen. The former tends toward a hyperglycemia and the latter, toward a hypoglycemia; and thereby there is a tendency to balance forces and regulate processes. It is possible that, in addition, insulin has a retarding influence on the conversion of liver glycogen to blood sugar, and epinephrine may have a similar retarding influence on the change from blood sugar to muscle glycogen. We shall see that

the situation is really more complex; for the pituitary and probably the adrenal cortex is also involved.

Estimations of Glycogen, Glucose, and Lactic Acid.—These three substances are of such fundamental importance in questions dealing with carbohydrate metabolism that brief reference to methods of determining them will be made.

Glycogen is quite resistant to the action of alkali but is easily hydrolyzed by acid. The tissue (the liver, for example) is boiled with alkali, filtered, and the glycogen in the filtrate precipitated with alcohol. The glycogen is hydrolyzed to glucose, and the amount of the latter determined, from which the amount of glycogen can be calculated.

The methods for determining glucose are many; some of them are based on the reduction of alkaline copper solutions (Fehling-Benedict solutions). The lactic acid may be determined by oxidizing it to acetaldehyde, the latter being distilled into an excess of sodium bisulfite, with which the aldehyde forms an addition product. The uncombined sodium bisulfite is titrated with iodine, from which can be calculated the acetaldehyde or lactic acid present.

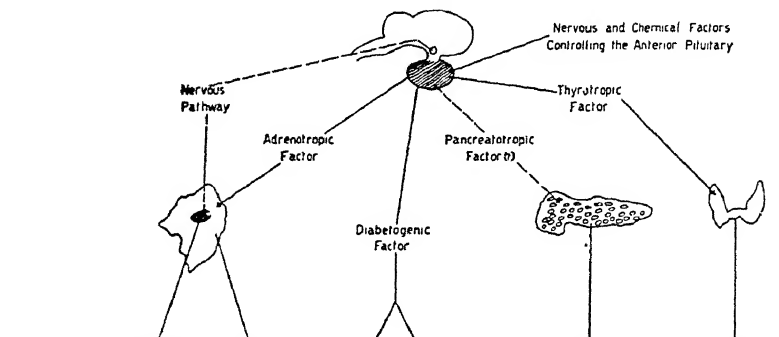
The Renal Threshold.—In the process of regulating the amount of sugar in the blood, we have discussed a number of factors at work: the amount of liver glycogen transformed to glucose; the amount of glucose itself transformed into muscle glycogen; and, somewhat indirectly, the amount of lactic acid converted into liver glycogen. We have seen that these factors are under the control of hormones. But the amount of glucose in the blood—as has already been indicated—also depends upon how fast the sugar is oxidized: in active muscular exercise, for example, the rapid breakdown of muscle glycogen would demand a rapid transfer of blood sugar to the muscles, and such sugar would then have to be replaced, probably by the further conversion of liver glycogen into glucose. The amount of sugar in the blood also depends upon how much carbohydrate is converted to body fat: the individual who eats much and exercise little will find this process of considerable importance.

There is, however, still another factor which has not yet been discussed; and this one involves the kidney. Normally, the amount of glucose in the urine is negligible. However, when for any reason, one or more of the various regulatory mechanisms become impaired and the amount of glucose in the blood increases appreciably above the normal, then the "renal threshold" is reached and appreciable quantities of glucose begin to appear in the urine.

Normally, the glucose in the blood filters through the glomerular membrane of the kidney and is again reabsorbed in the tubules. But when the sugar in the blood reaches, say, 140 mg. per cent, and above, the reabsorptive capacity of the tubules may be too highly taxed and sugar may pass into the urine. By using phloridzin, which prevents the reabsorption of the sugar, glucose readily passes into the urine and an experimental glycosuria is established.

Insulin.—This hormone will be discussed elsewhere (Chap. 24). However, since insulin is so intimately involved in carbohydrate

metabolism, an account of its influence must be given here. The extirpation of the pancreas in an animal gives rise to diabetes, whereby the sugar of the blood is increased above the normal amount (hyperglycemia) and sugar appears in the urine (glycosuria). Apparently, one important regulatory factor in maintaining the sugar of the blood at a constant level has broken down. This factor is due to a hormone, manufactured by the pancreas, to which the name "insulin" has been given. Banting, Best, Macleod, and Collip were able to extract a fraction from the pancreas which, when injected into a depancreatized animal, lowered the amount of blood sugar. They even showed that the



SECRETION →	EPINEPHRINE	CORTIN			INSULIN		THYROXIN
SITE OF ACTION →	LIVER AND TISSUES	LIVER AND TISSUES(?)	TISSUES	LIVER	LIVER	TISSUES	ALL TISSUES INCLUDING LIVER
ACTION →	Increases glycogen breakdown. Redistributes formed carbohydrate	Increases liver glycogen and blood sugar	Inhibits carbohydrate utilisation.	Increases carbohydrate production.	Decreases carbohydrate production	Increases carbohydrate utilisation	Increases rate of oxidation in tissues and rate of glycogen formation in liver.

Fig. 86.—The endocrine control of carbohydrate metabolism. (Long, *Sigma Xi Quarterly*, **26**, 175)

injection of such an extract into a normal animal lowers the blood sugar below the normal amount—so much so, in fact, that the animal may go into convulsions and die. This is a dramatic illustration of the importance of keeping the blood sugar at certain levels.

What, then, is the function of this insulin?* The injection of insulin into the depancreatized animal enables more sugar to be burned and more glycogen to be deposited. There is an actual increase in the amount of glycogen in the liver, and the glycogen in the muscle may also be increased. That oxidation is increased is discovered by determining the respiratory quotient (R. Q.) (see Chap. 20). This is a value obtained by dividing the volume of carbon dioxide eliminated by the volume of oxygen consumed. The rise in R. Q. to a figure usually approaching unity is an indication that carbohydrates, rather than fats or proteins, are being burned in the body. So that we may say that the function of insulin is a double one: it influences directly the

* See appendix, p. 570.

oxidation of carbohydrate, and it also regulates the quantity of glycogen deposited in the liver and in the muscle.

In connection with the relationship of insulin to carbohydrate metabolism, it should be mentioned that the injection of insulin causes a decrease in the amount of serum phosphate but an increase in the hexose monophosphate of muscle. As will be seen presently (p. 322), hexose phosphates are formed prior to the further break-down of carbohydrates, and a source of such phosphate is in the blood (which, therefore, shows a decrease).

Other Hormones Involved in Carbohydrate Metabolism.—The effect of hormones on carbohydrate metabolism is summarized in Fig. 86.

The removal of the pancreas from a dog is fatal; the animal dies in one to two weeks. The cat usually survives not more than five to six days. If, however, the pituitary is also removed from such a depancreatized animal, the survival period is increased to some twenty-two days in the cat, and the blood sugar is markedly decreased (from some 347 mg. per 100 cc. to 190). Somewhat similar results are obtained when the adrenals are removed from a depancreatized animal (Table 46).*

TABLE 46.—THE SURVIVAL AND EXCRETION DURING FASTING OF GLUCOSE, NITROGEN, AND ACETONE BY VARIOUS CATS. (Long, *Harvey Lectures*, Ser. 32, Williams and Wilkins Co., Publishers.)

Type of animal.	Number.		Survival.	Urine.				Blood sugar. *
				Glucose.	Nitrogen.	Acetone.	D† / N	
			days	*g/k/d**	*g/k/d**	*mg/ k/d**		*mg/ 100 cc.*
Depancreatized....	22	Average	5.3	3.2	1 3	116	2.7	347
		Range	2–12	1.6–6.4	0.8–2.2	28–275		212–788
Hypophysectomized-depancreatized ...	19	Average	22.0	0.4	0.7	5	0.6	190
		Range	8–85	0.0–1.5	0.3–0.9	0–27		16–355
Adrenalectomized-depancreatized...	18	Average	14 0	0.6	0.6	13	1.0	186
		Range	5–28	0.0–1.6	0.3–0.9	3–48		13–362

* Signifies gm. or mg./kilo/day.
† D = glucose; N = nitrogen.

The modification of the diabetic stage by a hypophysectomy has been shown by Houssay to be due to the absence, more specifically, of the anterior pituitary. The "antagonistic action" of the hormones—the insulin with its tendency to lower blood sugar and the pituitary with its tendency to increase it—is probably not a very exact picture of what actually happens. We know that there is a "diabetogenic

* It should, however, be added that Grollman, from the results of his own work, denies that the adrenals—that is the cortex—"is preeminently involved in the maintenance of carbohydrate metabolism."

hormone" in the anterior pituitary—a hormone which tends to increase the amount of sugar in the blood and which tends to decrease the production of insulin in the pancreas.

By injecting dogs with relatively large quantities of this diabetogenic hormone over a number of days, Young has shown that the diabetes may continue indefinitely after treatment with the extract has ceased.

The pituitary also contains an "insulinotropic hormone" (or "substance")—one which stimulates the production of insulin

A relationship somewhat similar to that existing between the pituitary and the pancreas has been postulated for the adrenals and the pancreas. Here it has been shown that the causative agent is not the adrenal medulla, containing the adrenaline (epinephrine), but the cortex. Adrenaline, as we have seen (p. 314), may be of some importance in carbohydrate metabolism, but some substance (or substances?) in the cortex appears even more important.

Carbohydrate Tolerance.—The ingestion of carbohydrate causes a temporary increase in the amount of sugar in the blood. In a normal individual, after the administration of from 1 to 2 gm. of glucose per kilogram of body weight, the blood sugar will increase in one hour from about 110 mg. per 100 cc. of blood to about 160 mg. At the end of 2 to 2½ hours the amount will be normal again. In a diabetic the increase is much more marked, and the return to normal is a slower process. For example, after an ingestion of glucose, the blood sugar of the diabetic may increase from 200 to 400 mg. in two hours, and at the end of the third hour it is still 300 mg.

Oxidation of Glucose in the Body.—We have seen how carbohydrates appear as glucose in the body, and we have seen how a number of mechanisms are necessary to regulate the amount of blood sugar. We must now discuss the further breakdown of glucose, whereby it is ultimately oxidized to carbon dioxide and water.

This oxidation occurs in the various tissues of the body. However, for the present, our information is largely confined to muscle, due to the fact that, so far, most of the important results deal with oxidation within the muscle.

In muscle, carbohydrate metabolism is the characteristic feature. So far it has not been possible to show that fats are oxidized here. Among amino acids (representing protein), glutamic acid, aspartic acid and alanine have been shown to be oxidized by muscle; and it is significant that their initial products are α-ketoglutaric, oxaloacetic and pyruvic acids, all three associated with the metabolism of carbohydrates (see pages 326, 327).

The experiments are usually performed on muscle (or other tissue) which has been either extracted, minced or sliced.

Extracts of muscle contain the substances necessary for the anaerobic breakdown of carbohydrate to lactic acid, but oxidation reactions involving molecular oxygen are not possible with such extracts. For the purpose of oxidation studies, minced tissues work well at times, but even better, on occasion, is the use of sliced tis-

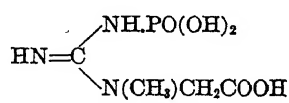
sues, with many cells still sufficiently intact to perform their normal functions.

Breakdown of Carbohydrates in Muscle.—It has long been known that one of the products of (anaerobic) muscular activity is lactic acid. During oxygen consumption, lactic acid disappears and glycogen reappears. During the contraction of muscle, lactic acid appears; and in the recovery process, the acid disappears. So that the muscle cycle is (*a*) anaerobic contraction: glycogen → lactic acid; and (*b*) oxidative recovery: lactic acid → glycogen. Meyerhof showed that the oxygen consumption accounted for the combustion of one-fifth of the lactic acid, and he also proved that with the disappearance of lactic acid, glycogen reappeared. One-fifth, then, of the lactic acid—or some equivalent—is oxidized to carbon dioxide and water, whereas four-fifths is resynthesized to glycogen.

A point of some importance has to be mentioned here. During recovery, with oxygen consumption, the R. Q. (respiratory quotient) is unity. This, as we have said before (p. 316), indicates the combustion of carbohydrate. But the relative proportions of carbon, hydrogen, and oxygen in lactic acid are the same as in carbohydrate; hence, the burning of lactic acid also yields an R. Q. of 1. In other words, from the R. Q. alone—and this is our chief guide at present—it is quite impossible for us to tell whether the oxidation is the result of the burning of part of the lactic acid, or whether all the lactic acid is resynthesized and the oxidation and heat production are due to the oxidation of some hexose, possibly an intermediate in the process: glycogen ⇄ lactic acid.

Iodoacetic Acid.—It has been believed that the contraction of muscle was the result of the conversion of glycogen to lactic acid, which liberated energy necessary for contraction. However, Lundsgaard proved that he could poison the muscle with iodoacetic acid, CH_2ICOOH, and it would still continue to contract for a short period, even though no lactic acid was produced. This meant that the process of contraction and the production of lactic acid were not inseparably connected. Soon the process of contraction was connected with another substance—phosphocreatine.

Phosphocreatine (also called *phosphagen* and *creatine phosphate*) was isolated by Fiske and Subbarow from protein-free muscle filtrate. They showed it to have the formula



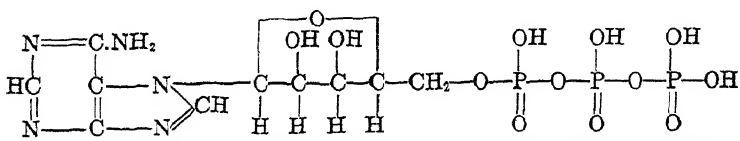
They, together with the Eggletons, proved that, during contraction, it was hydrolyzed and lost its phosphate group; and that, during recovery, it was resynthesized.

A muscle poisoned with iodoacetic acid contracts so long as there is phosphocreatine as such; when this compound breaks down completely, contraction ceases. Here, unlike normal muscle, there is no resynthesis of the phosphorus compound.

To explain these facts, it was assumed that, normally, the energy for contraction is derived from the breakdown of phosphocreatine. At this stage, glycogen is converted to lactic acid. During recovery, when most of the lactic acid is resynthesized to glycogen, some of the energy produced (resulting from the oxidation of lactic acid or its equivalent), is utilized for the resynthesis of the phosphocreatine.*

Other Phosphates in Muscle.—It is important to realize that the conversion of glycogen to lactic acid involves the production of a number of "intermediate" products. In fact, this phase of muscle chemistry has borne rich fruit during the past few years, resulting in the isolation of a number of substances from muscle, and in their identification as esters of organic compounds with phosphoric acid.

Fresh frog or rabbit muscle, which has been cooled to $-1°$ C. and minced, can be extracted with water, and the extract, cell-free, will convert glycogen to lactic acid. This conversion involves complicated reactions, as we know today, and it is clear that such an aqueous extract contains not one enzyme but many enzymes. If we dialyze such a muscle extract it becomes inactive. In the dialysate we find, among other things, adenosinetriphosphate, which is now known to play a very active part in muscle metabolism. It is a coenzyme Adenosinetriphosphate is related to adenylic acid (p. 86): the difference between the two is that the former contains three phosphoric acid groups, whereas the latter contains but one such group.



Adenosinetriphosphate. (Also called adenylpyrophosphate and ATP.)

It was Lohmann who, in 1929, isolated from fresh muscle the barium salt of a compound which, on hydrolysis at the neutral point, gave pyrophosphoric acid, $H_4P_2O_7$, and adenylic acid. That the compound in muscle is adenosinetriphosphate was made reasonably clear by the fact that on the addition of adenylic acid to muscle juice, phosphate disappeared and pyrophosphate appeared.

Adenosinetriphosphate (ATP) is involved with phosphocreatine in the process of muscle contraction, which includes the loss and gain of a phosphate group. ATP reacts with the myosin of muscle (p. 446)—now known to be not only a protein but an enzyme—whereby the ATP

* In phosphocreatine—as in adenosine-triphosphate (above)—we find "energy condensed in phosphate bonds. . . . The average derivable from hydrolytic decomposition of these energy-rich phosphate bonds is 11,000 cals. . . . The 11,000 cals. condensed in the energy-rich phosphate bond (represented by ~ ph) represent a biological energy unit. Migrating continuously from compound to compound, the quantity ~ph in many respects can be regarded as largely independent of the compound to which it is attached . . . " (Lipmann).

Adenylic acid (p. 86) is primarily responsible for the distribution of this unit (~ph).

The phosphocreatine and adenylpyrophosphate, etc., are to be distinguished from many phosphate esters, such as hexose phosphate, in which the average energy of the ester linkage is 3,000 cals.

is hydrolyzed to adenosinediphosphate (ADP) and inorganic phosphate. Because of this reaction the enzyme myosin is also known as adenosinetriphosphat*ase*.

In the conversion of glucose to hexose phosphates, ATP is also involved (p. 323).

The adenosinediphosphate (ADP) may lose still another phosphate group and be converted to adenylic acid (adenosinemonophosphate). This is accomplished by another enzyme, adenosinediphosphatase—about which less is known than about myosin.

The myosin is believed to be responsible for the contractile and elastic properties of muscle.

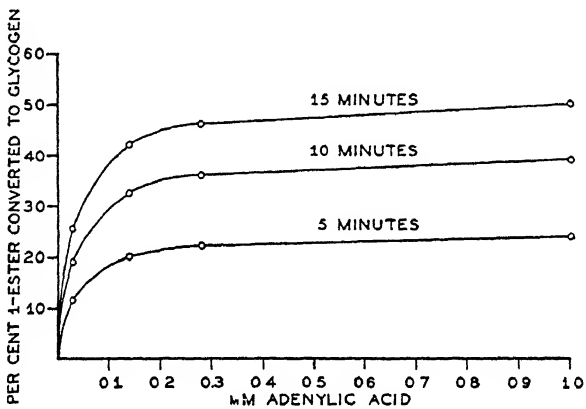
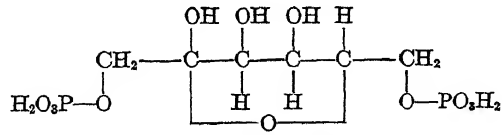


Fig. 87.—Effect of adenylic acid on glycogen synthesis in a dialyzed enzyme preparation of calf brain. The reaction mixture contained 15 mM glucose-1-phosphate, 100 mg per cent of glycogen, 0 05 M veronal buffer of *p*H 6.9, and varying amounts of adenylic acid. The period of incubation was 5, 10, and 15 minutes, as marked on the curves. The temperature was 25°. (Cori and Cori, *J. Biol. Chem.*, **135,** 735.)

In resting muscle, hydrolysis and synthesis of ATP proceed continuously, with no appreciable change in concentration. In working muscle, there is a decrease in the concentration of ATP, which is restored to its original level only after rest.

Another important phosphate is hexose joined to phosphoric acid. When fluoride is used in the place of iodoacetic acid to poison muscle, lactic acid fails to appear, as might be expected, but there is also an absence of free phosphoric acid. From this "fluoride" muscle a fructose diphosphate has been isolated:



Fructose diphosphate.

When mixed with an extract of muscle, this diphosphate will produce lactic acid just as well as if glycogen were used.

The Breakdown and the Synthesis of Glycogen in Animal Tissues. As we have seen, the end product of glycogen breakdown in the liver is glucose, and the end product in muscle is lactic acid. However, we do know that the initial stages in glycogen degradation are identical in both these tissues, and presumably also in other tissues.

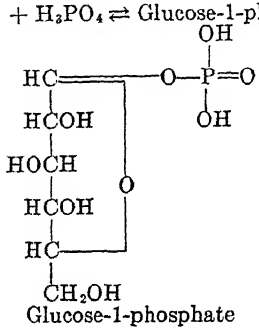
The first step in the degradation of glycogen is a reversible process

If extracts from liver, heart, muscle, brain, yeast or potato are dialyzed (to remove inorganic and organic phosphates and coenzymes) the residue can no longer decompose glycogen; but if inorganic phosphate and adenylic acid are added, glycogen is decomposed quite rapidly.

The enzyme in these extracts primarily responsible for the breakdown of glycogen has been given the name *phosphorylase.*

The phosphorylase, with adenylic acid as coenzyme, catalyzes the reaction.

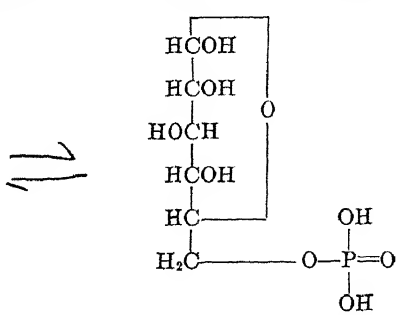
$$\text{Glycogen} + H_3PO_4 \rightleftarrows \text{Glucose-1-phosphate.}$$



Glucose-1-phosphate

The reaction is a reversible one (Fig. 87).* At equilibrium, the concentration of inorganic phosphate is some 80 per cent, and that of organic phosphate (glucose phosphate), 20 per cent.

The reaction proceeds to the right because another enzyme, *phosphoglucomutase* converts glucose-1-phosphate to glucose-6-phosphate.



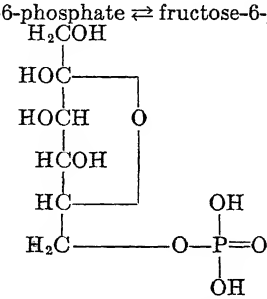
Glucose-6-phosphate

This reaction is also reversible. It is very much accelerated by the presence of magnesium ions.

* By the action of liver, heart or brain phosphorylase on glucose-1-phosphate, a product behaving in all respect like glycogen has been obtained. If muscle phosphorylase is substituted, the product formed, strangely enough, resembles starch.

Still another enzyme, *isomerase* (phosphohexoseisomerase), catalyzes this reaction:

Glucose-6-phosphate ⇄ fructose-6-phosphate



Fructose-6-phosphate.

At equilibrium, there is about 80 per cent of the glucose ester and about 20 per cent of the fructose ester (1).

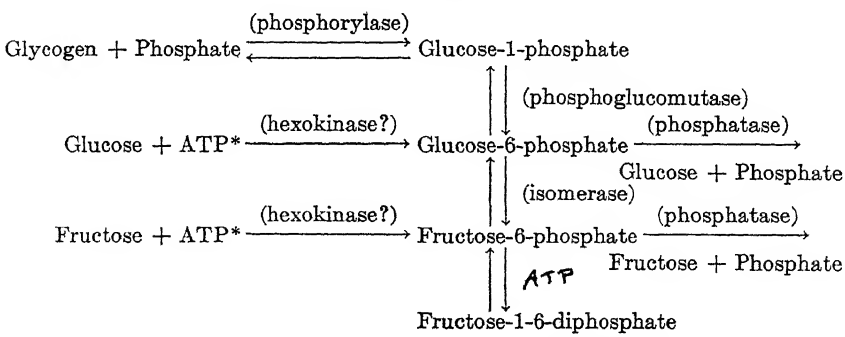
This mixture of the two esters is a normal constituent of resting muscle. In muscle, as glycogen disappears, the mixed ester begins to accumulate. In liver, however, much more glycogen disappears than is accounted for by the appearance of hexose-monophosphate.

Using a liver extract, both glucose-1- and glucose-6-phosphate are converted to glucose and inorganic phosphate. In contrast to muscle, liver contains an enzyme, *phosphatase*, which acts on the hexosemonophosphate formed from glycogen by the phosphorylase.

It is, then, the combined action of phosphorylase and phosphatase (possibly isomerase too?) which is responsible in the liver for the conversion of glycogen to glucose.

Turning back to muscle, it has been shown that the fructose part of the equilibrium esters (1) is converted to fructose-1-6-diphosphate (p. 321) before the latter is changed to lactic acid.

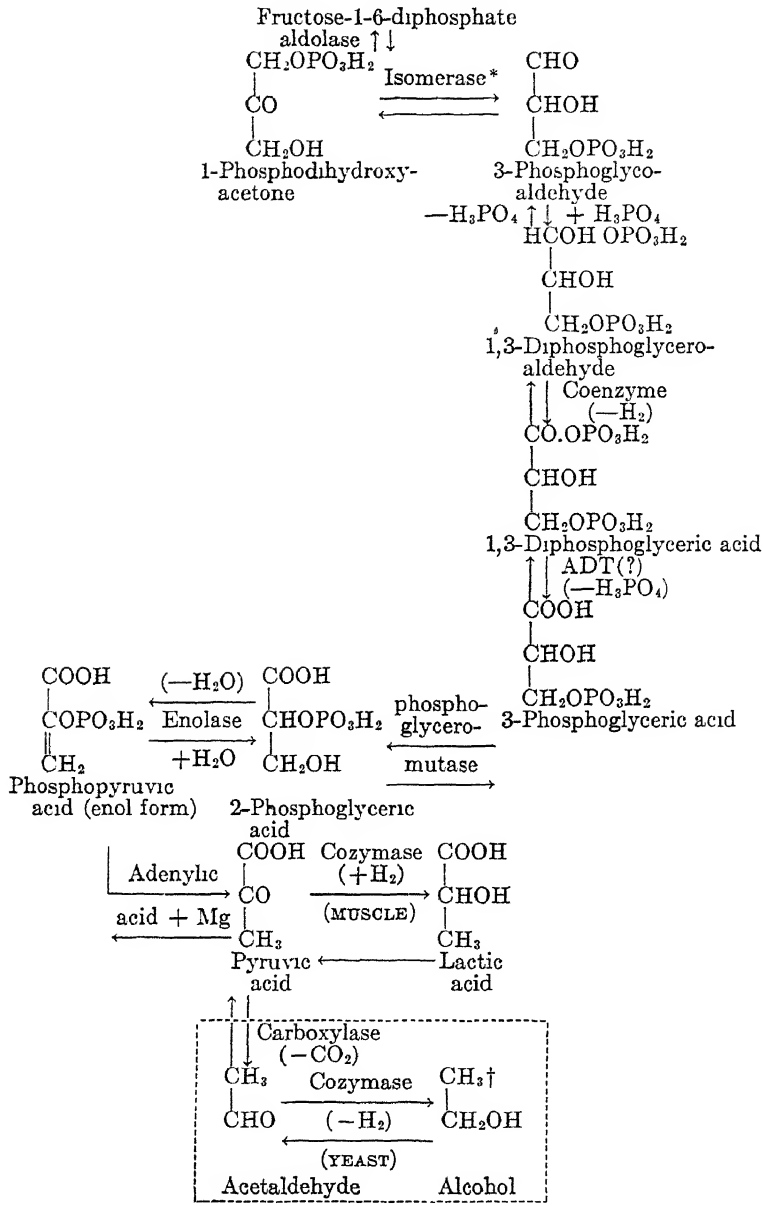
To summarize these and other reactions:



The conversion of fructose-6-phosphate to fructose-1-6-diphosphate is made possible by a supply of phosphate from adenosinetriphosphate (ATP).

Glycogen to Lactic Acid.—We have just discussed the conversion of glycogen to hexosediphosphate.

* ATP = Adenosinetriphosphate (Phosphate donor).



* "Triose phosphate isomerase (or more briefly isomerase) is an enzyme which catalyzes the reaction glyceraldehyde phosphate ⇌ dihydroxyacetone phosphate. The transformation of hexosediphosphate into triose phosphate is brought about by the joint action of two enzymes, called together zymohexase; namely, aldolase, which splits the hexose ester into 1 glyceraldehyde phosphate + 1 dihydroxy-acetone phosphate, and isomerase (3). In the absence of isomerase, living cells would be able to metabolize only *half* of the hexose molecule by way of glyceraldehyde phosphate to pyruvic acid and its various end-products (lactic acid in muscle, alcohol and CO_2 in yeast, etc.)." Meyerhof.

† (In alcoholic fermentation, the pyruvic acid instead of being reduced to lactic

Using enzyme preparations obtained from such a muscle extract, and trying first one and then another likely "intermediate" compound, various esters of phosphoric acid have been formed. For example—and only a few examples in this complicated field can be given—a dialyzed muscle extract acting on hexosediphosphate (containing, among other things, fructose diphosphate) produces phosphodihydroxyacetone. When dialyzed muscle is used, phosphocreatine cannot be split into creatine and phosphoric acid unless adenylic acid is present; then the latter is converted to ATP. Still using this muscle extract, it can be shown that two molecules of phosphopyruvic acid react with adenylic acid to yield two molecules of pyruvic acid and one molecule of ATP. Then again, Meyerhof and Lohmann, two very active workers in this field, have shown that dialyzed muscle (or, for that matter, a yeast extract, which, in some ways, resembles muscle extract) contains an enzyme, to which the name *aldolase* has been given, which brings about typical aldol condensations between phosphodihydroxyacetone and various aldehydes to give ketophosphoric acid. The suggestion has, therefore, been made that in the breakdown of hexosediphosphate, dihydroxyacetone phosphate and glyceraldehyde phosphate are formed.

From such—and many more—experiments, a possible scheme outlining the changes in muscle contraction has been evolved. It should be said, in view of past experience, that such a scheme is "subject to change without notice." One scheme is as shown on page 324.

The scheme represents phosphoric acid being transferred from one compound to the other—from hexose, through the various trioses, until we reach pyruvic acid, when the molecule of phosphoric acid is given to adenylic acid.

The lactic acid formed, according to this scheme, by the interaction of pyruvic acid and triosephosphate, is synthesized back again into glycogen (at least, this is true of four-fifths of the acid).

The Oxidation of Carbohydrate.—We have seen, in dealing with muscle, that during the process of contraction, glycogen is converted to lactic acid, and during relaxation, oxygen is absorbed, carbon dioxide is produced and four-fifths of the lactic acid synthesized to glycogen. During this stage, an amount of material is oxidized which is equivalent to one-fifth of the lactic acid produced; but we are not at all sure that it is the lactic acid which is further oxidized.

However, assuming that the substance oxidized is lactic acid, how is this oxidation brought about? What are the intermediate steps in the change from lactic acid to carbon dioxide and water?

The citric acid theory of Krebs (one of a number of theories) derived from work on isolated tissues, suggests that an intermediate compound containing six carbon atoms is citric acid (or a close deriva-

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acid, is decarboxylated, that is, it loses carbon dioxide and is changed to acetaldehyde, which is further reduced to ethyl alcohol:

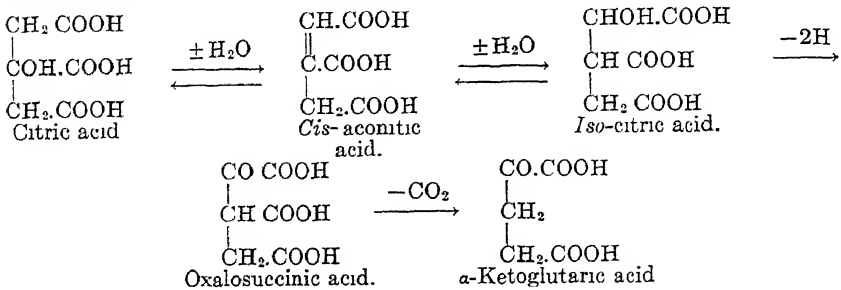
$$CH_3CO.COOH \xrightarrow{CO_2} CH_3CHO \xrightarrow{+H_2} C_2H_5OH.)$$

tive); and is derived from the combination of one molecule of oxaloacetic acid and pyruvic acid.

Citric acid is found in common foods and is excreted in the urine in small quantities. Little citrate is recovered when administered; which means that much of it is metabolized and changed.

Of the various substances tried, large excretions of citric and α-ketoglutaric acids are obtained when certain dicarboxylic acids are injected—such as succinic, fumaric, malic, and oxalacetic

Evidence has been obtained to show that citric acid is first oxidized in the body to α-ketoglutaric acid by the following paths:



The oxidation of citric acid to α-ketoglutaric acid can be shown by adding sodium arsenite to the tissue—a substance which poisons the enzymes responsible for the further oxidation of α-ketoglutaric acid.

That α-ketoglutaric acid is further oxidized, and to succinic acid, can be shown by adding α-ketoglutaric acid to the tissue in the presence of malonic acid—a substance which destroys the enzyme responsible for the further oxidation of succinic acid.

That the oxidation proceeds still further beyond the succinic acid stage has also been experimentally verified.

The various stages can be summarized thus:

Citrate → α-ketoglutarate → succinate → fumarate → malate → oxaloacetate

Not only is citric acid oxidized in the body, but it can also be synthesized. For example, citrate is formed when oxaloacetic acid is added to muscle tissue. This reaction requires the addition of two carbon atoms to the oxalacetic acid. It is generally assumed that the carbohydrate derivative ("triose") supplying these additional atoms is pyruvic acid itself.

Krebs has formulated the citric acid theory of muscle oxidation by assuming that oxalacetic acid and pyruvic acid combine to form *cis*- aconitic acid. From then on the changes are as shown in Fig 88.

It will be noticed that the regenerated oxalacetic acid combines with a fresh quantity of pyruvic acid to restart the citric acid cycle.

During this cycle, pyruvate is consumed (explaining the oxidation of some of the lactic acid), carbon dioxide is eliminated—the loss of CO_2 will be noticed in several steps during the process—and water is also eliminated (as a result of the loss of hydrogen in several steps during the process; this hydrogen eventually combines with oxygen, see p. 396).

The amino acids, alanine, aspartic acid and glutamic acid, shown on the side lines in Fig. 88, are connecting links between carbohydrates and proteins.

α-ketoglutaric acid can also be formed from the condensation (by enzymes) of oxaloacetic acid with β-keto acids to form citric acid. Since the β-keto acids are oxidation products of fats, a link between the metabolism of carbohydrates and fats is established.

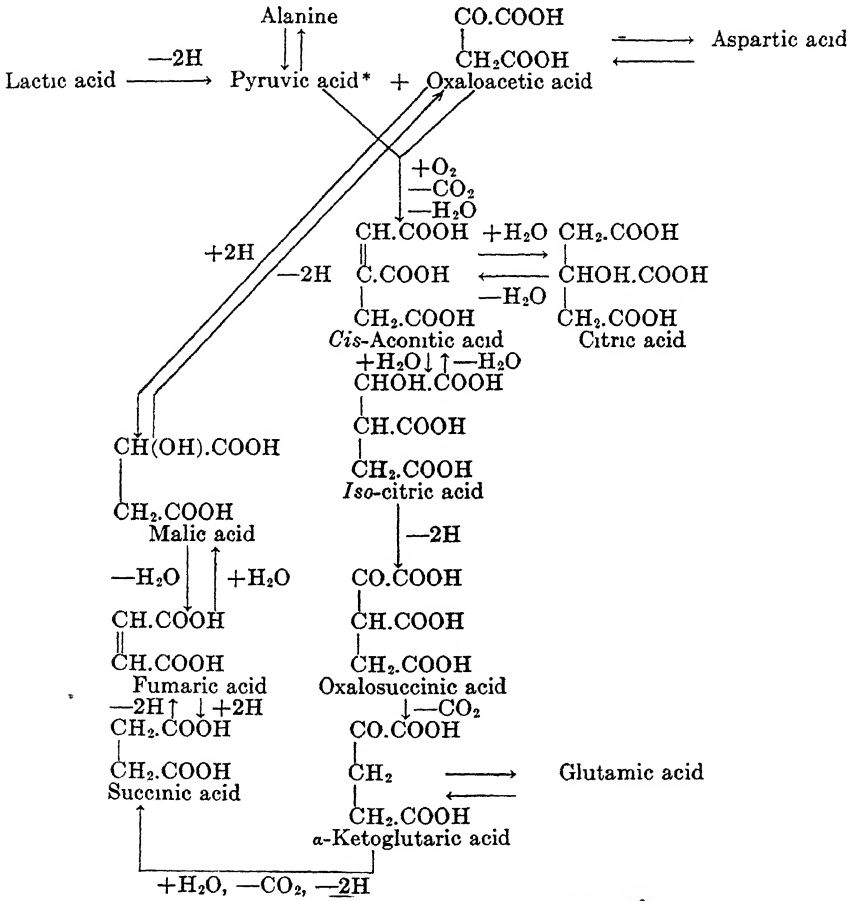


Fig. 88.—Oxidation of carbohydrate (Krebs).

Utilization of Carbon Dioxide.—In a general way it may be said that plants can build up their nutritional requirements from such simple compounds as carbon dioxide, water and ammonia (in the presence of light energy), and that by contrast the animal must be provided with amino acids, certain fatty acids, vitamins, etc. The need for carbon by the plant is met by carbon dioxide; in the animal, by various organic substances. Until quite recently, carbon dioxide was regarded as purely an end product of metabolism.

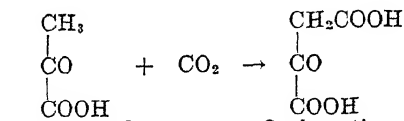
* More recent evidence suggests that the acetyl group, derived from pyruvic by decarboxylation, combines with oxalacetate to form *cis*-aconitate.

In autotrophic unicellular organisms, with no photosynthetic mechanism, carbon dioxide can also serve as the sole source of carbon. Heterotrophic bacteria, it has been shown more recently, can utilize carbon dioxide, but their carbon needs cannot be met by this compound alone. This has also been shown to be true of animals Carbon dioxide, then, even in animals, can no longer be looked at as a mere end product of metabolism.

The main reaction in animals involving carbon dioxide is a carboxylation, or a fixing of the carbon dioxide as the carboxyl group of a dicarboxylic acid. This is an analogous reaction to the "dark reaction" in photosynthesis (p. 206):

$$R.H + CO_2 \rightleftarrows R.COOH$$

The central metabolic product in carbohydrate metabolism, pyruvic acid, is converted to dicarboxylic acids (oxaloacetic, α-ketoglutaric and succinic acids) with the help of carbon dioxide; for example,



Pyruvic acid. Oxaloacetic acid.

The fact that CO_2 can enter directly into combination with pyruvic acid to form, among other things, oxaloacetic acid, suggests then still another method—other than that from malic acid—for the production of oxaloacetic acid (see Fig. 88)

Wood and Werkman, working with propionic acid bacteria, and using radioactive carbon or the stable carbon isotope, C^{13} (in the form of CO_2), as the "fixing agent," suggested that a three-carbon compound, like pyruvic acid, combines with carbon dioxide to form oxaloacetic acid, which is then reduced to succinic acid. Radioactive carbon was present in the molecule of succinic acid

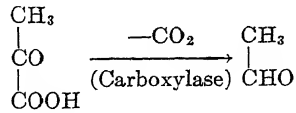
Evans and Slotin showed that in the presence of pigeon liver in a bicarbonate buffer, radioactive carbon dioxide and pyruvic acid could be used to form α-ketoglutaric acid. All the radioactivity of the acid was confined to the carboxyl group α to the carbonyl oxygen. This is an example of the utilization of inorganic carbon for the building of a dicarboxylic acid.

It should be made clear that in reactions of the kind we have discussed in this chapter, not only is the attempt made to isolate the intermediate products, but attempts to identify the oxidizing mechanisms involved are also made. The subject of such mechanisms is relegated to a separate chapter (Chap. 19).

Thiamine and Carbohydrate Metabolism.—Among vitamins, thiamine, or vitamin B_1, stands out as being very much involved in the metabolism of carbohydrates. Along these lines Funk did some pioneer work. Peters showed that pigeons suffering from a deficiency of thiamine were unable to oxidize pyruvic acid, which, as we have

already seen, is a highly important intermediate compound in the metabolism of carbohydrates.

That thiamine joined to phosphoric acid, is a coenzyme for carboxylase (p. 540) suggested, for a time, a convenient explanation, for it was said that carboxylase converts pyruvic acid to acetaldehyde:



but that it cannot perform this chemical action without the presence of the coenzyme, co-carboxylase (the phosphorylated thiamine). This is all perfectly true, except that a reference to Fig. 88 (p. 327) will show that such is not the metabolic path of pyruvic acid—in muscle, at least. Whether the process is more peculiar to the brain—and Peters' work was done with brain tissue—remains to be seen.

At any rate, the important observation still remains: in the absence of thiamine the body is unable to oxidize pyruvic acid.

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CHAPTER 17

METABOLISM OF THE LIPIDS

As has already been stated elsewhere (Chap 3), under the "lipids" we include true fats, phospholipids, and the sterols. We shall take up each of these in turn.

THE FATS

Energy is stored very largely in the body in the form of its fats. It is not a "passive storage," as was at one time supposed, but is constantly being broken down and restored, the "turnover" being accomplished in several days (p. 334).

We have also seen (p. 118) that certain unsaturated fatty acids are "essential" food constituents. Just why they are "essential" is not known. Are they of the character of vitamins which supply needed groups for the building of biologically important enzymes?

It seems fairly clear that an important part of the fat molecule, the fatty acid, is transported towards its ultimate destination in the form of a phospholipid molecule (as, for example, some of the fatty acids in lecithin). This is of especial interest, since it serves as a connecting link between the metabolism of carbohydrates, which are also transported as phosphorylated compounds, and fats.

The fatty acids, mainly in the form of phospholipids, constitute one-half of the dry substance of the brain and are found in abundance in liver, lungs, adrenals and kidneys. In at least one of these organs, the liver, the functions of these fats, or rather the phospholipids, is becoming clearer (p. 334).

We have already seen (Chap. 12) that the fats are hydrolyzed in the small intestine, resynthesized in the walls of the intestine and absorbed via the lacteals into the lymph. The fat finds its way into the blood stream through the thoracic duct. The absorption of some 60 per cent of the fat can be accounted for in this way. What happens to the rest is not at all clear. It is assumed that the rest must be absorbed directly into the portal circulation, in a manner similar to the carbohydrates and amino acids.

In any case, after a meal rich in fat there is a substantial increase of fat in the blood. Such an increase becomes noticeable in one to three hours after a meal, and the increase may continue up to six or seven hours, when a decrease sets in, and this continues until the normal level is reached again.

What happens to the fat after it has reached the blood is not clear. Some of it, for a short time at least, is deposited in adipose tissue, liver, etc.; some of it undergoes oxidation in the tissue cells. But how is such an oxidation accomplished? And through what inter-

mediate compounds does the fat pass before it is ultimately eliminated from the body as carbon dioxide and water?

We shall discuss these debatable topics presently. But brief reference must here be made to two theories which, until quite recently, have had an important place in discussions dealing with the metabolism of fat. One theory we owe largely to Bloor, who, as a result of the analyses of the phosphatids of blood cells during fat absorption, came to the conclusion that the fats (or rather, the fatty acids) before being oxidized, are first converted to phosphatids; for the increase in the latter more or less paralleled the increase in fat.

The other theory dealing with the metabolism of fat we owe to Leathes, who claimed that a certain part, at least, of the oxidation of fats occurs in the liver Leathes pointed to the relatively high content of unsaturated fatty acids in this organ, and emphasized the fact that unsaturated fatty acids are more readily oxidized—at least *in vitro*—than saturated ones. He drew the conclusion that desaturation was a preliminary process in the oxidation of fat, and that such desaturation occurred in the liver.

Bloor's views on the importance of phospholipids as intermediates in fat metabolism are generally accepted; Leathes' views, however, have not gone unchallenged.

The Storage of Fat.—The body stores fat very largely in three places: subcutaneous connective tissues; abdominal cavity; and intermuscular connective tissue. An analysis of such fat reveals that it is mainly composed of the glycerides of stearic, palmitic and oleic acids; although smaller quantities of other acids (lauric, myristic) may also be present. The stored fat may have its origin in any one of the three common foodstuffs, carbohydrate, protein and fat; or it may be derived from a mixture of them.

The tendency is for a production of fat characteristic of the animal. The body fat, in other words, shows differences from the fat of the food. However, it is possible to change the nature of such stored fat by a radical change in the diet. In one experiment; the melting point of the body fat of a dog on a normal diet was 20° C. By feeding mutton tallow, the melting point was increased to 40° C., and by substituting linseed oil, the melting point was reduced to 0° C.

Aside from change in melting point, another easily available means of detecting chemical changes in the composition of fats is a determination of the iodine number. Feeding soy bean oil to rats yielded a body fat with an iodine number of 132; and replacing the soy bean oil with cocoanut oil lowered the iodine number to 35 (Anderson and Mendel).*

It had always been supposed that the storage of fat occurred during "surplus" periods: that when the body could not burn its foodstuffs fast enough, the excess, for the time being, was stored away as body fat. With this view in mind, it followed that on an insufficient diet no

*Livestock producers are able to "harden" (p. 33) animal body fats by feeding the animals a ration high in carbohydrate or protein and low in oils containing unsaturated fatty acids.

such storage should take place. The work of Schoenheimer (and his co-workers, Rittenberg and Graff) has completely upset this view.

Isotopes in Intermediary Metabolism.—Schoenheimer's work is of such fundamental importance, and has so many possible ramifications, that a brief discussion of its nature is necessary. The key to his work is the application of "heavy" hydrogen to investigations dealing with intermediary metabolism. The "heavy" hydrogen is the "label": once it is properly attached to the compound under examination, the latter can be fed, and its course in the body traced by determining this heavy hydrogen in the various tissues.

Ordinary hydrogen, whether obtained from organic or inorganic sources, is a mixture of isotopes—a mixture of hydrogen (atomic weight 1) and "heavy" hydrogen, or deuterium (atomic weight 2). The latter is present to the extent of 0.02 per cent. By treating oleic acid with hydrogen (in the presence of a suitable catalyst such as nickel) we get stearic acid:

$$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH \xrightarrow{H_2} CH_3(CH_2)_7.CH_2.CH_2.(CH_2)_7.COOH$$

If, however, we treat the oleic acid with deuterium instead of hydrogen, we get

$$CH_3(CH_2)_7.\underset{D}{CH}.\underset{D}{CH}.(CH_2)_7.COOH$$

Chemically, this acid cannot be distinguished from stearic acid. Nor is the body able to distinguish it—which is indeed a great advantage, for the body treats it in presumably the same way as the normal acid. However, the presence of heavy hydrogen in the molecule makes identification simple. In the position in which deuterium finds itself in the molecule of fat, it is "fixed"; which would hardly be true if the deuterium were to replace the hydrogen of the carboxyl group.

Two types of isotopes are available: the natural isotopes of H, O, N, C and S—the products, very largely, of the work of Urey—and many artificially produced radioactive modifications.

One method of procedure with isotopes is first to prepare the isotopic compound, and then to administer it to the animal. The isotopic content of organs (or their constituents) is finally determined. If the injection of compound A containing deuterium gives rise to a compound B with a deuterium content higher than that of the body fluids, then we may say that B is derived from A.

In protein metabolism, involving amino acids (p. 349), the carbon chain and the amino groups are considered separately, since they follow different metabolic paths. Deuterium or the carbon isotope is used for the carbon chain and N^{15} for the amino group.

The analytical procedure for testing the products varies. If the isotope is radioactive, the radioactivity may be measured by the Geiger counter. For the analysis of deuterium, the compound is burned

and the density of the heavy water produced is determined, or the amount of deuterium in the hydrogen present is measured in the mass spectrometer.

The mass spectrometer is used for the measurement of isotopes in general.*

In the experiment dealing with fat storage, Schoenheimer fed mice some linseed oil which had been partially hydrogenated with deuterium. The fat in the diet amounted to 1 per cent, and the total diet offered was insufficient for normal development: the animals lost weight. It was supposed that, with such a diet, the animals would burn the fat quite rapidly, and, therefore, show no fat deposits. At the end of a four-day period, the animals were killed, the fat of the fatty tissues isolated, and the deuterium determined.

The animals had consumed 251 mg. of fat ("labeled" with deuterium). From the fat depots an amount of deuterium corresponding to 119 mg., or 47 per cent, was recovered. The rest was burned to carbon dioxide and water. Obviously, then, a large part of the fat, even when given in insufficient amounts, is first deposited in the tissues rather than burned directly.

The popular idea that body fat "stays put" for a long time, provided there is no starvation, must be revised. Using adult mice, it has been found that one-half of their fatty acids is regenerated in 5 to 9 days.

This comparatively rapid process of fat regeneration applies equally well to the phospholipids: in both cases there is a continual breakdown and building up of fats and phospholipids.

Cholesterol and sterols in general are also regenerated, but at a slower rate (from fifteen to twenty-five days).†

The Liver and Fat Metabolism.—A large part of the absorbed fat finds temporary storage in the liver. The liver is a depot not only for excess fat (for the time being) but, as we have seen, for excess carbohydrate (in the form of glycogen). It is believed by many that in this organ much of the fat is changed to phospholipid (lecithin, etc.) before it undergoes further metabolic changes.

Interesting in this connection is the fact that in the absence of choline, one of the constituents of lecithin, fat accumulates in the liver to an abnormal extent (p. 335)—an abnormality which can be removed by including choline in the diet. Using labelled phosphorus, Chaikoff showed that ingested choline speeds up the rate of "turnover" of phospholipid in the liver. It would seem that lecithin is necessary for active metabolic changes of the fats.

The liver has another important function in so far as fats are concerned: it converts long-chain fatty acids into C_4 compounds, the

* The phrase "atoms per cent" of isotope has been introduced. Examples will make this clear. Glycine containing 10 atom per cent N^{15} means that the nitrogen of the glycine molecule contains 10 per cent more N^{15} atoms than the normal glycine; or 10.37 per cent of its nitrogen is N^{15}. Valeric acid with 10 atom per cent deuterium means that 10.02 per cent of all of its hydrogen atoms are present as deuterium (D).

† We speak of a rapid or slow "turnover" of foods or metabolites.

"acetone bodies" (p. 341), which are now regarded as "normal" products of the metabolism of fatty acids

That the liver plays an important rôle in the metabolism of fat has been known for some time. In poisoning due to phosphorus or chloroform, the amount of fat in the liver is much increased. Where excess food in the form of fat is given, relatively large quantities of fat concentrate in this organ. The same is true during starvation, when the fat reserves of the body are called into play. In either case, the total fatty acid content may increase from 3 to 20 per cent.

Using Eck-fistula dogs—dogs having an artificial communication between the portal vein and the vena cava—it can be shown that there is a very marked drop in the amount of total fatty acids and cholesterol in the serum. The animals continue to lose body fat due to the inability of the body to synthesize sufficient quantities of fat.

It was also believed for a time that the fats—or rather, the fatty acids—prior to their oxidation undergo a preliminary desturation in the liver, a view based largely on the relatively high iodine number of liver fats (Leathes). The discovery by Burr that certain unsaturated fatty acids are "essentials" in the diet—at least, in so far as the rat is concerned—(Chap. 7) has led many to question the correctness of this hypothesis. And yet mammals can desaturate fatty acids, though whether this occurs more specifically in the liver is not yet clear. Schoenheimer and Rittenberg have shown this to be true by feeding mice with a diet containing 10 per cent of deuterostearic acid (that is, in this case, a linoleic acid which was hydrogenated with deuterium). At the end of some twelve days, the animals were killed, the total fatty acids of the carcass isolated, and these acids were next fractionated into saturated and unsaturated acids. Both fractions contained deuterium in combination. The animal, then, has the ability to convert a saturated into an unsaturated acid.

But, to complicate the situation, the organism can also accomplish the reverse process: the conversion of unsaturated to saturated fatty acids. By feeding an unsaturated fatty acid, containing deuterium in its molecule, both saturated and unsaturated deutero-fatty acids were isolated.

However, neither linoleic nor linolenic acid (p. 29) is formed by desaturation; which may explain why such acids, needed by the body, must be supplied with the food.

Stetten points out that the quantity of fat (in the form of fatty acids) in the liver is the result of a number of factors: amount received from the diet (Fig. 89, *a*, *b*); amount degraded (*c*); amount transported from liver to depot storage (*d*); and amount transferred from depot to liver (*e*).

Choline and Fat Metabolism.*—Normally, the liver contains from four to five per cent of fat. Under certain abnormal conditions, this figure may increase to as much as 30 per cent and more; which may mean that some one-half of the total weight of the liver is fat.

* For choline in nutrition, see p. 171.

The amount of fat in the liver of an animal may be very readily increased by placing the animal on a diet rich in fat. Another method—and this has also been known for some time—is to include relatively large amounts of cholesterol in the diet offered the animal. In either case, there is produced experimentally a liver abnormally rich in fat—comparable to what is often observed as a result of pathological changes which affect this organ. Best and Channon proved that the addition of lecithin to the diet prevented this abnormal increase of fat in the experimental animal. A closer investigation revealed the fact that the causative agent is choline (p. 36), a constituent of the lecithin molecule.

The amount of choline in the liver seems to remain constant under varying conditions. Choline may be supplied in the diet or it may not; fatty liver may be present or it may not: the amount of choline still remains the same.

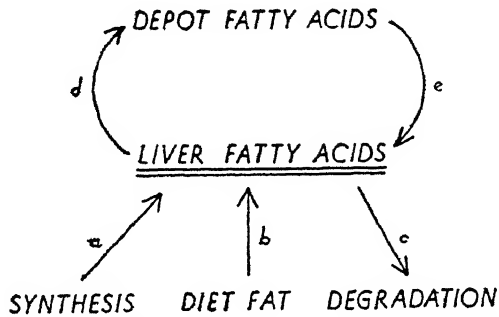
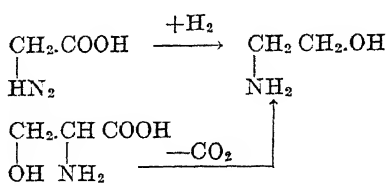


Fig. 89.—The sources and fates of liver fatty acids. (Setten, Jr. and Salcedo, *J. Biol. Chem.*, **156**, 27.)

The answer to this problem is that choline can be synthesized in the body, and in amounts to correspond with the needs of the body provided, of course, sufficient precursors are available. This synthesis is accomplished by a combination of methyl groups (obtained from methionine) with ethanolamine, $OH—CH_2—CH_2—NH_2$, which the animal can produce by the reduction of glycine, or perhaps by the decarboxylation of serine:



The lack of choline also gives rise to an acute renal hemorrhage. It would seem, however, that this disease is not due specifically to choline, but rather to a lack of some essential substance containing a labile methyl group; for the choline content of rats with this hemorrhagic disease is equal to, if not slightly more than, that of normal rats. Even the hemorrhagic kidneys contained more choline than normal kidneys.

The research connected with choline and its effect on fat metabolism has an interesting historical background. Soon after the discovery of insulin, Allan observed that despite adequate treatment with insulin, depancreatized dogs fed on lean meat, sucrose, and bone ash did not survive beyond a few months. He noticed a disturbed function of the liver, due to a fatty infiltration, and he made the important discovery that this fatty infiltration could be prevented by the addition of raw pancreas to the diet. This suggested to the author the possibility that the pancreas contained a hormone other than insulin of importance in the normal functioning of the liver.

Somewhat later Hershey discovered that lecithin could replace the raw pancreas in the diet of depancreatized dogs; and then Best showed that, in general, such fatty infiltration of the liver could be cured by the addition of choline. The conclusion drawn from such experiments was that the efficacy of raw pancreas in Allan's original experiments was primarily due to the presence of choline (as part of the lecithin or lipid fraction of the pancreas). Nevertheless, Dragstedt has given reasons for the assumption that in this case we are not dealing with choline, but with a new hormone, to which the name *lipocaic* has been given.

Dragstedt finds, in the first place, that the beneficial effect of feeding pancreas is specific, in the sense that other organs, such as the liver and the brain, are quite inert, though they contain more choline than the pancreas. Furthermore, fat-free alcoholic extracts of pancreas have been obtained which contain neither lecithin nor choline and which are active.*

The research on choline in connection with the prevention and cure of fatty livers—the name *lipotropic* has been given to describe this action of choline—was greatly facilitated by the use of isotopes. Chaikoff, using radioactive P as a "tracer," found choline to accelerate the rate of formation of phospholipids in the liver. Stetten fed choline containing heavy N and detected a high concentration of the isotopic N in choline isolated from the bodies of the animals. Du Vigneaud fed methionine containing deuterium in the methyl group to immature rats on a diet devoid of methionine and choline. The creatine and choline isolated from the bodies of these animals contained a high concentration of the isotope.

Du Vigneaud had previously shown that on a diet devoid of methionine and cystine, rats did not improve when homocystine (p. 55) was added, but became quite normal when offered a mixture of choline and homocystine. This made probable the ability of the body to utilize the methyl group of choline in converting homocystine to methionine.

Such information, added to that obtained from the isotopic studies, emphasized the importance of "transmethylation" in the body: the ability of the body, for example, to utilize the methyl group of choline in order to transform homocystine to methionine; and to utilize, if

* Chaikoff, etc., maintain that the active fraction in raw pancreas is *not* lipocaic (see reference at end of the chapter).

necessary, the methyl group of the latter to build up choline from simpler substances such as ethanolamine (see p. 336).

It should be made clear that there are various types of fatty livers. For example, fatty livers produced by phosphorus or carbon tetrachloride poisoning cannot be cured by choline. Nor can cures with choline be accomplished with fatty livers produced by the use of anterior pituitary extracts.

Blood Fat and Fatty Acids.—A small amount of fatty acids is found in blood in the form of neutral fat. Appreciable quantities of these fatty acids are found incorporated in the phospholipids (lecithin, etc., see p. 332). While in the corpuscles there is found cholesterol as such, in the plasma from 60–70 per cent of the sterol is found in ester combination with fatty acids.

The neutral fat in the plasma may range up to (about) 370 mg. per 100 cc. The fat content (and fatty acids in general) are usually increased during fasting or on a meat diet exclusively, suggesting an unusual demand on fat in the absence of carbohydrate. Increases have also been noticed in other instances: narcosis, alcoholism, following the administration of chloroform and phosphorus, diabetes, etc. In hyperthyroidism there is usually a decrease of fat and fatty acid in plasma.

The Oxidation of Glycerol.—The oxidation of fat probably involves, first, the hydrolysis of the molecule into glycerol and fatty acid, and then the oxidation of the two components. There is reason to believe that the intermediate steps in the oxidation of glycerol resemble those in the oxidation of carbohydrates. Glycerol, like glucose, gives rise to glycogen in the liver, though, it is true, not to the same extent. Again, in diabetes produced by means of phlorhidzin, glycerol is practically quantitatively converted to glucose. So that in all probability, the glycerol, through such stages, perhaps, as glyceraldehyde, pyruvic acid or dihydroxyacetone, is finally broken down to CO_2 and water (see Chap. 16).

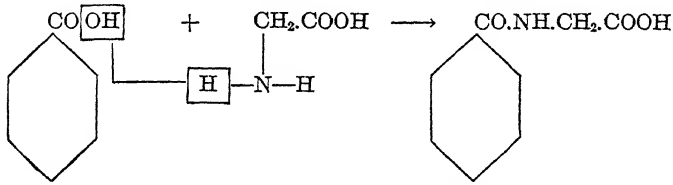
The Oxidation of Fatty Acids.—The intermediate stages through which a fatty acid passes as it is changed from, let us say, a C_{18} acid to CO_2 and water, are known very imperfectly. "Acetone bodies" are produced in the liver and these have been traced to the breaking down of fatty acid. These "acetone bodies" are β-hydroxybutyric acid, $CH_3.CHOH.CH_2.COOH$, acetoacetic acid, CH_3COCH_2COOH, and acetone, CH_3COCH_3. The first two are undoubtedly partial oxidation products of butyric acid, and the acetone may be regarded as derived from the acetoacetic acid by the loss of carbon dioxide.

It will be noted that while there is in this chapter a discussion of possible intermediates in fat metabolism, nothing is said about mechanisms, about the role played by various enzymes in these processes (compare with chapter 16). The reason is that little is known.

Lehninger has stressed the fact that during *in vitro* studies with liver cells, if such cells are ruptured, they can no longer oxidize fatty acids. Within certain limits, oxidation can proceed in such cells in the presence of adenosinetriphosphate (p. 320).

The first important contribution to the problem of the metabolism of fatty acids was made by Knoop.

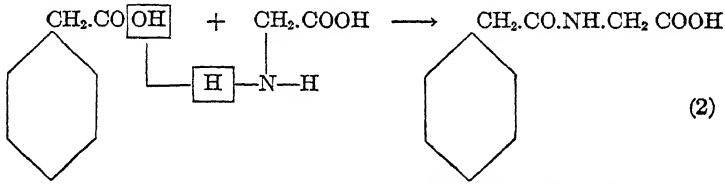
Knoop's β-Oxidation Theory.—While straight-chain compounds are, as a rule, very readily oxidized by the organism, this is not true of compounds containing the aromatic nucleus. Knoop combined various fatty acids with the phenyl group, fed the resulting phenylated compounds, and investigated the products eliminated in the urine. Benzoic acid yielded hippuric acid; which meant that the benzoic acid combined first with the glycine supplied by the body:



(1)

Hippuric acid.

Phenylacetic acid produced phenylaceturic acid:



(2)

Phenylaceturic acid.

With higher fatty acids, the products isolated were either hippuric acid (1) or phenylaceturic acid (2). For example,

$C_6H_5.CH_2.CH_2.COOH$ yields (1)
$C_6H_5.CH_2.CH_2.CH_2.COOH$ yields (2)
$C_6H_5\ CH_2.CH_2.CH_2.CH_2.COOH$ yields (1)
$C_6H_5.CH_2\ CH_2.CH_2.CH_2.CH_2.COOH$ yields (2); etc.

We would complicate the procedure by pointing out that the yield is very far from 100 per cent, or that a considerable quantity of such phenylated compounds combine with glucuronic acid, as well as with glycine, and will merely state the conclusions drawn by Knoop from these experiments. His results, he argued, indicated that oxidation of fatty acids occurred in such a way that at each stage in the degradation process there was a loss of two carbon atoms, due to oxidation at the β-carbon atom. For example,

$$C_6H_5.\overset{\beta}{C}H_2.\overset{\alpha}{C}H_2.COOH \rightarrow C_6H_5COOH$$

$$C_6H_5.CH_2.\overset{\beta}{C}H_2.\overset{\alpha}{C}H_2.COOH \rightarrow C_6H_5CH_2COOH$$

$$C_6H_5.CH_2.CH_2.\overset{\beta}{C}H_2.\overset{\alpha}{C}H_2.COOH$$

$$C_6H_5.\overset{\beta}{C}H_2.\overset{\alpha}{C}H_2.COOH$$

$$\downarrow$$

$$C_6H_5.COOH$$

In other words, no matter what fatty acid derivative we start with, as a result of such β-oxidation, with the resulting loss of two carbon atoms at each stage, the final products must be either benzoic acid or phenylacetic acid; and such acids, by combination with glycine, will be eliminated as hippuric and phenylaceturic acids, respectively.

All these views apply equally well to the naturally occurring fatty acids.

While the natural fats are even-carbon compounds, it does not necessarily follow that odd-carbon fats are not utilized by the body. As a matter of fact, rats have been shown to utilize such odd-carbon fats (made synthetically) for growth and depot fat formation.

A striking confirmation of the two-carbon oxidation comes from the work of Schoenheimer and Rittenberg. These authors have shown that deuteropalmitic acid (C_{16}) can be isolated from mice fed with deuterostearic acid (C_{18}). These mice were fed for five days with deuterostearic acid, and the palmitic acid, isolated from their carcasses, contained appreciable quantities of deuterium.

ω-Oxidation.—It should also be pointed out that aside from the theory of β-oxidation, an omega (ω) theory has established itself rather firmly in recent times. This newer theory does not do away with the older one; it merely acts in a supplementary manner. Verkade, Flaschenträger, and others, have succeeded in isolating dicarboxylic acids from urine after feeding fatty acids to their animals. It has suggested to them that the terminal methyl group may be first oxidized to the carboxyl group, and that then β-oxidation proceeds from both ends. For example,

$$\begin{array}{rcl} CH_3.CH_2\,CH_2 & & .\ CH_2.CH_2.COOH \\ & \downarrow & \\ COOH.CH_2.CH_2 & \ldots\ldots & CH_2.CH_2.COOH \\ & \downarrow & \\ COOH & \ldots\ldots & COOH \end{array}$$

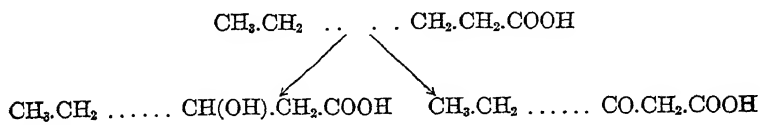
Even numbered, saturated dicarboxylic acids are utilized by the organism; but the utilization is more complete the longer the chain length of the acid. The C_{16} and C_{18} acids are practically completely oxidized.

Multiple Oxidation.—Deuel has shown that butyric acid (C_4) and caproic acid (C_6) yield the same amount of acetoacetic acid in the urine (representing "acetone bodies," p. 338), but that caprylic (C_8) yields twice as much of the acid—an indication of a split into two fragments of C_4. Higher acids (palmitic, oleic and stearic) gave three of the C_4 fragments.

Quastel offered the theory (of multiple alternate oxidation) which suggests that not only does oxidation occur at the β carbon atom, but, *at the same time,* probable oxidations occur at each alternate carbon atom in the chain, resulting in the formation of chemically simple two- and four-carbon products.*

* Medes offers evidence to show that C_4 compounds (ketone bodies) are formed by condensation of a 2-carbon intermediary resulting from the β-oxidation of the fatty acid. The C_4 compounds, in other words, are not directly formed from the higher fatty acids.

Oxidation of Fatty Acids.—Assuming the β (and other) theories of the oxidation of fatty acids, much still remains to be learned. There is the probability—judging from what has already been said—that a gentle oxidation process begins at the β-carbon atom:



Assuming a mechanism of this sort, with regular breaks whereby two carbon atoms are split each time, we eventually arrive at acetoacetic acid as one of the products. There is evidence that this acid can be converted into acetic acid in the tissues. According to one view, acetoacetic acid is converted to acetic acid which, under normal conditions, proceeds to CO_2 and H_2O. However, if too much acetic acid is formed, a resynthesis of acetoacetic acid takes place, giving rise to "acetone bodies."

It may be mentioned here that Knoop visualized his β-oxidation in the following form:

$$\mathrm{R.CH_2.CH_2.COOH} \xrightarrow{-\mathrm{H_2}} \mathrm{R.CH{=}CH.COOH} \xrightarrow[-\mathrm{H_2}]{+\mathrm{H_2O}}$$

$$\mathrm{R.CO.CH_2.COOH} \xleftarrow{+\mathrm{H_2O}} \mathrm{R.COOH + CH_3COOH}$$

Ketone or Acetone Bodies.—The fact that in starvation and in diabetes acetone bodies (β-hydroxybutyric acid, acetoacetic acid and acetone) accumulate to an appreciable degree has led to the view that they are abnormal metabolic products. This view must be revised, for the evidence is accumulating that these substances are indeed normal metabolic products.

These acetone bodies are produced by the liver. However, the liver cannot, but the muscles can utilize them. Wherever possible, carbohydrate is the source of energy of the liver. If however, such carbohydrate is largely lacking—as in starvation—or if it cannot be properly utilized—as in diabetes—then the liver turns its attention to fats and amino acids, a number of which produce these acetone or "ketone" bodies.

Under such conditions, the production of ketone bodies may be so large that the muscles are unable to metabolize much of them. Such a situation brings on a "ketonuria"—an excessive elimination of acetone bodies in the urine.

"Fats burn only in the presence of carbohydrates" is a picturesque but, as we now know, a misleading expression. It was assumed that in the absence of carbohydrates, fats are incompletely burned and therefore produce acetone bodies; and that one function of carbohydrate was to facilitate the oxidation of fats. Carbohydrates, it was maintained, inhibited ketosis, or an excessive formation of acetone bodies, by increasing their utilization (ketolysis).

This, however, is probably not the case. The present point of view

—not altogether without some opposition—is that so long as carbohydrates are present, the liver utilizes them for metabolic purposes; but when they are absent, or present in insufficient amounts, this organ is forced to utilize other metabolites which give rise to excessive quantities of acetone bodies.

Carbohydrates, according to this point of view, inhibit ketosis, not by increasing the utilization of acetone bodies, but by keeping the production of these bodies down to a minimum (antiketogenesis).*

It has already been mentioned that in diabetes these acetone bodies are produced in excessive quantities. The injection of insulin not only causes a fall in the blood sugar (p. 314) but a marked drop in the output of acetone bodies. In accordance with the theory just outlined, this action of insulin can best be explained on the ground that the hormone induces the retention of glycogen in the liver; and that, whenever possible, the liver will utilize carbohydrates in preference to fats or proteins, or metabolites obtained from them.

In support of this view, it is known that the liver of the diabetic is poor in glycogen. Furthermore, the intravenous injection of large amounts of dextrose to depancreatized dogs, *receiving no insulin*, results in a rapid drop of acetone bodies both in the blood and in the urine.

Conversion of Carbohydrate into Fat.—Such a conversion has been common knowledge for a long time. The fattening of animals on a diet with little fat but much carbohydrate, and the experience of men and women on such a diet, make the conclusion inevitable. Adult, fasted rats, fed high carbohydrate (or high protein) diets, synthesize large quantities of fat.

Schoenheimer, in a continuation of his ingenious experiments on intermediate metabolism, involving deuterium as an "indicator," has shown that on a diet of bread, and practically devoid of fat, with heavy water as a source of deuterium, mice not only convert carbohydrate (and presumably some protein) into fat, but, almost as rapidly, break down such fats, to be replaced by a fresh supply.

Using mice, the deuterium content of the fatty acids of the body rose rapidly for a short period and then remained stationary. The animals did not change in weight, which means that the total amount of fat remained constant; and therefore, side by side with the synthesis of fatty acid, there must have taken place a decomposition of a corresponding amount of fatty acid.

These experiments indicate that the conversion takes place on a normal diet, and not necessarily on an excessive diet; and they emphasize the eternal cycle, within the body, of alternate synthesis and analysis of its structural units.

How are we to explain, in chemical terms, the conversion of carbohydrate into fat? There is little beyond guesswork. It has been assumed by some that pyruvic acid, a probable intermediate in the metabolism of carbohydrate, is acted upon by carboxylase, an enzyme present in a

* For opposition to this view, see, for example, Bobbitt and Deuel, *J. Biol. Chem.*, **143**, 1 (1942).

number of tissues of the body, the function of which is to attack α-ketonic acids and split off CO_2:

$$CH_3.CO.COOH \rightarrow CH_3.CHO$$

It is next assumed that the acetaldehyde may undergo an aldol condensation:

$$CH_3.CHO + CH_3.CHO \rightarrow CH_3.CH(OH).CH_2.CHO$$

giving rise to a four-carbon compound. By a number of such additions of acetaldehyde, the C_{16} and the C_{18} compounds would be reached. From then on, desaturation, oxidation, and reduction would eventually lead to a naturally occurring fatty acid.

$$\begin{array}{ll} CH_3 & \ldots CH(OH).CH_2.CHO \\ & \downarrow \\ CH_3 & \ldots CH{=}CH\ CHO \\ & \downarrow \\ CH_3 & \quad CH{=}CH.COOH \\ & \downarrow \\ CH_3 & \ldots CH_2.CH_2.COOH \end{array}$$

Another theory postulates that the condensation is one between pyruvic acid and acetaldehyde, liberating CO_2.*

Conversion of Fat into Carbohydrate.—The situation here is not at all clear. There is some evidence that rat liver slices have the ability to transform fatty acids into carbohydrate.

The Conversion of Protein into Fat.—Using phloridzinized or diabetic animals, and feeding them protein, an increased excretion of glucose in the urine is obtained. Glutamic acid, arginine, glycine, and the like, are "glycogen formers." We know, of course, that carbohydrates can be converted into fats; and now we see that amino acids—obtained from protein—can be converted into carbohydrate. This also means that certain amino acids, in turn, may be converted into fat by first being transformed into carbohydrate.

There are some amino acids which are not "glucose formers." Using either leucine, tyrosine or phenylalanine, and perfusing them through a liver, gives rise to acetoacetic acid—which is also a direct product of fat metabolism. Hence it would appear that some amino acids do not necessarily have to pass through the intermediate carbohydrate stage before being converted into fats.

Longenecker has shown that there can occur a synthesis of fatty acid (and therefore fat) not only from carbohydrate but also from protein. Fasted rats, fed high carbohydrate or high protein diets, synthesize large quantities of fat.

Effect of Vitamins on the Formation of Fat.—From the extensive work of McHenry and Gavin it would seem that protein cannot be converted into fat unless pyridoxine as well as thiamine is present.

It has long been known that thiamine plays an important rôle in carbohydrate metabolism, primarily in connection with the formation

* There is some indication that higher aliphatic alcohols may be intermediates in fat metabolism.

of pyruvic acid, a substance which might be utilized for the metabolism of fatty acids.

The conversion of carbohydrate into fat may require the presence not only of thiamine, but of at least two other factors of the vitamin B complex, riboflavin and pantothenic acid.

It is possible that the vitamins function as follows:

$$\text{Protein} \xrightarrow{\text{Pyridoxine}} \text{Carbohydrate} \xrightarrow[\text{Riboflavin} + \text{Pantothenic acid}]{\text{Thiamine} +} \text{Fat}$$

However, much of all this is largely hypothetical.

Obesity.—In the vast majority of cases, obesity results from the consumption of too much food (carbohydrate and protein as well as fat), from the comsumption of food "above the calorific requirements of the body "(Newburgh). Obesity resulting from endocrine disturbances is rare.

PHOSPHOLIPIDS

Lecithin.—The neutral fats have always been regarded as sources of fuel for the body. Lecithin—and, more broadly speaking, the phospholipids—is an essential structural element of the cell: phospholipids are present in every cell of the body. Their possible function as "intermediates" in the metabolism of fat has already been referred to. Sinclair is of the opinion that one of the fatty acids of the triglyceride molecule is replaced by the phosphoric-acid-base complex of the phospholipid, that the fatty acid is desaturated, and that both are a means toward "rendering the fatty acids readily diffusible and combustible." This same author fed elaidic acid (the stereoisomer of oleic acid) in the form of its glyceride, elaidin, and found that one-third of the total fatty acids in the phospholipids of the liver (and skeletal muscles) was made up of elaidic acid; which is evidence that the phospholipid is involved in the intermediary metabolism of fat.

But all this tells us nothing of the intermediate metabolism of lecithin (and phospholipids). The truth of the matter is that we know practically nothing. Apart from the small intestine, King has shown lecithinase to be present in the kidney, liver, and other organs. This would make it appear that, as in the case of neutral fats, lecithin is first hydrolyzed before it is further oxidized. Under such conditions, we get glycerol, fatty acid, phosphoric acid, and choline. The fate of the first two we have already discussed. The phosphoric acid is needed in cell metabolism, in bone formation, etc. The choline, as we have already seen, is of importance in regulating the amount of fat in the liver; and, as we shall see presently (Chap. 25), in the form of acetylcholine, it is of importance in the activity of the nervous system.

The organism has the ability to synthesize phospholipids. The eggs of ducks and hens raised on a diet low in lipid showed more of this substance than was taken in by the food. Rats on synthetic diets, con-

taining fat but devoid of phospholipid, grow quite well. Since lipids are essential cell constituents, we must postulate the synthesis of these constituents within the body.

In the attempt to secure information relative to phospholipid metabolism, Perlman, Ruben, and Chaikoff prepared radioactive phosphorus by bombardment of ordinary phosphorus with deuterons. The phosphorus so obtained was dissolved in aqua regia, and a solution of disodium phosphate prepared. The phosphate salt was fed to rats, the animals were killed at various intervals, the lipids in the tissues were extracted, and the "labeled" phosphorus (in the phospholipid) determined by means of a Geiger counter.

"Labeled" phospholipid appeared in various tissues. Two phases in phospholipid metabolism were observed: formation (or deposition) and utilization (or removal). A sharp increase in labeled phospholipid content was shown by liver and intestine, while carcass, kidney, and brain showed a slower rise. The rate of disappearance of labeled phospholipid from these tissues decreased in the following order: liver, intestine, and kidney. No decline was observed in the carcass (and possibly the brain).

Choline has the ability to increase the rate of phospholipid metabolism in the liver. The amount of phospholipid is rapidly increased, but also rapidly removed.

In blood the phospholipids appear to be transported in combination with the proteins. These lipid-protein combinations seem to be quite common. They occur in cell nuclei, cell membranes, chloroplasts, egg yolk, milk, etc. The thromboplastic factor (p. 269) is probably a lipid-protein complex.

Cephalin.—Of cephalin, the phospholipid closely allied, at least chemically, to lecithin, we know little. According to Chargaff, one important physiological function can be definitely assigned to it: "it is the prosthetic group in the specific protein, the thromboplastic factor, which is the natural activator of blood coagulation."

Sphingomyelin.—In the Niemann-Pick disease, characterized by the deposition of phospholipids in the spleen, liver, brain, etc., Klenk showed that the chief lipid in these organs was sphingomyelin. Significantly enough, the amounts of lecithin and cephalin were within normal ranges. This means, in other words, that in this disease there is some disturbance in the metabolism of sphingomyelin.

Phospholipids in Blood.—The usual (normal) values given for phospholipids in blood (including not only lecithin but cephalin and sphingomyelin) range from 2.5 to 14.5 mg. per 100 cc. for plasma, and 12 to 25 mg. per 100 cc. for red blood cells. These figures are in terms of "lipid phosphorus." In the form of "total phosphatide" the "lipid phosphorus" figures are multiplied by 23.5.

There is a definite increase in lipid phosphorus in the blood after the ingestion of fat, an increase which continues for some hours.

Increases also occur in several diseases, notably in diabetes. Decreases have been noted in several febrile conditions.

CEREBROSIDES

These substances are abundant in brain matter, but practically nothing is known of their metabolism. An interesting discovery, however, is that in Goucher's disease, which is characterized by large lipid deposits in various tissues, the predominant substance present is phrenosin (p. 38), one of the cerebrosides.

CHOLESTEROL

We know little concerning the metabolism of cholesterol in the body. It is an essential constituent of all cells; and it, again like lecithin, can be synthesized by the body. Cholesterol is excreted in the form of coprosterol in the feces after hydrogenation by intestinal bacteria (p. 252).

That cholesterol is synthesized in the body has been known for some time (Knudson; Schoenheimer). With deuterium as the isotope, Bloch and Rittenberg have since shown that so simple a substance as acetic acid is used in the synthesis of this sterol. Feeding sodium deutero acetate to mice and rats led to the formation of deutero cholesterol. The isotope was shown to be present both in the side chain and in the nucleus.

Bloch and Rittenberg have also made another highly important contribution, still using the isotope technique: bile acids (p. 226) can be formed from cholesterol. This brings cholesterol one step nearer in its relationship to other steroids in the body.

Cholesterol in Blood.—In the erythrocytes most of the cholesterol is in the free state. In the plasma, some 20 to 40 per cent is in the free state and the rest in the form of an ester (cholesterol joined to fatty acid).

The normal range of (total) plasma cholesterol (free plus combined) varies from 140 to 230 mg. (in very approximate figures).

Hypercholesterolemia—increased amounts of cholesterol is found in a number of pathological cases; diabetes is an outstanding one. The plasma cholesterol in this disease runs roughly parallel with the total fatty acids. Rabinowitch goes so far as to claim that the concentration of plasma cholesterol is in some ways even more important than that of blood sugar.

While the total cholesterol may increase several hundred per cent in diabetes, the proportion of free cholesterol to cholesterol ester remains fairly constant.

In diseases of the kidney, hypercholesterolemia is common; values often reach up to 700 mg. per 100 cc.

Still another instance of increase in cholesterol is to be found in jaundice, in which there is an obstruction of the bile duct. The increase in the sterol runs hand in hand with the hyperbilirubinemia, such increases disappearing with the removal of the obstruction.

High figures for cholesterol are found in hypothyroidism, such figures being roughly in inverse ratio to the basal metabolic rate (p. 411) a low metabolic rate indicating relatively marked hypercholesterolemia.

Atherosclerosis, a senile form of arteriosclerosis, has often been associated with hypercholesterolemia, but with little evidence that such association is justified.

Sharp decreases in the amount of cholesterol in blood (hypocholesterolemia) often occur in pernicious anemia. Fifty mg. per 100 cc. are not uncommon in infectious diseases and in hyperthyroidism. In the last instance, the amount of cholesterol is roughly in inverse ratio to the values found for the basal metabolic rate (p. 411).

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CHAPTER 18

METABOLISM OF PROTEINS

THE proteins are first hydrolyzed in the digestive tract into amino acids. Proteins of a more complicated kind also give rise to amino acids, but, in addition, produce other substances. For example, casein splits off phosphoric acid; nucleoprotein yields nucleic acid, which hydrolyzes further (Chap. 5). But the amino acids are certainly the essential constituents of the protein molecule.

These amino acids, of which there are some 21, are absorbed through the lumen of the small intestine, pass into the portal system, and thence to the liver. Some of these amino acids pass on to the tissues to form tissue protein; others are utilized for the formation of specific substances (glutathione, bile salts, certain hormones, etc.); others are deaminized.* The deamination—the loss of the amino group—takes place largely in the liver and in the kidney.

As a result of this deamination, the amino group, split off as ammonia, contributes to the formation of urea, and the deaminized portion may be oxidized, ultimately to carbon dioxide and water, or it may form glucose, or it may form fatty acid, or it may be resynthesized into the amino acid.

Since the amino acids have, in common, a structure involving the α-amino carboxylic acid, one general reaction for deamination probably serves for all of them. The exceptions are the "basic" amino acids (such as lysine), with more than one amino acid group; it is not yet clear whether, in such a case, deamination affects the second amino group. Also, once the ammonia is split off, the method by which urea is formed certainly cannot vary for different amino acids.

However, the oxidation of the deaminized portions of amino acids raises many difficulties. It is, of course, obvious that, having removed the NH_2 groups from the amino acids, we are left with residues which, chemically, resemble one another no more closely than the original substances. These deaminized residues may form glycogen (and therefore glucose) or fatty acid; or they may be oxidized. Since there is good reason to suppose that a partial oxidation at least is necessary in the first two instances, a study of the steps in this oxidation process becomes extremely important. But, unfortunately, the variety of structures among the amino acids is such that few oxidative reactions can be applied to all of them. In almost all cases it is necessary to study the oxidation of each individual acid.

In the course of the changes which proteins undergo in the body, some 80 per cent of the nitrogen belonging to such proteins is excreted in mammals as urea. In the normal, adult individual, the nitrogen

* What probably occurs most frequently is that tissue proteins are broken down to amino acids and that these are then deaminized.

intake, as represented by the protein intake, is approximately equivalent to the nitrogen output, as represented by the excretion of urea and other nitrogenous substances. The body is said to be in "nitrogen equilibrium."

As a result of these various changes which proteins undergo in the body, not only do we find various "end-products" of protein catabolism in the urine, but most of these products first appear in the blood. These substances include urea, ammonia, uric acid, creatinine, amino acids, etc.

The Formation of Tissue Protein.—Theoretically, the formation of tissue protein is the reverse of a hydrolytic process: it is the building up of a protein from the amino acids. We find tissue proteins—proteins actually manufactured in the body—in all the organs; yet we have only the vaguest ideas regarding their method of formation. It has been suggested by some that the proteolytic enzymes which so readily hydrolyze proteins are available, under certain conditions, for synthetic work.

Of the various tissue proteins, those which have been most studied are found in blood: hemoglobin, fibrinogen, prothrombin, serum albumin and serum globulin. In various ways, the liver, the bone marrow, the spleen and the intestine are involved in the formation of one or more of these proteins; the liver stands out predominantly.*

Plasma proteins, representing the albumin and globulin fractions, are manufactured in the liver to a large extent. A dog with an Eck fistula—a dog whose portal vein is connected with the vena cava, therefore preventing blood from the intestines from entering the liver—has little capacity to form blood protein when compared with the normal dog. Furthermore, a marked lowering in the amount of blood protein, a hypoproteinemia, is observed in cirrhosis of the liver.

The capacity of the body to regenerate plasma protein has been shown by Whipple, who first developed a hypoproteinemia by his method of plasmapheresis, wherein the blood plasma is depleted by bleeding the animal and then returning the washed red corpuscles in physiological salt solution (0.9 per cent). When such animals are fed various foods containing protein, a marked increase in plasma protein occurs.

Weech arrived at the same result by inducing hypoproteinemia in his animals with diets low in protein and then adding the "test substance" rich in protein. Beef serum, egg white and casein, in decreasing order of efficiency, are prominent "regenerators" of plasma protein.

There is also much evidence that fibrinogen and prothrombin are largely manufactured by the liver. By excision of a portion of the liver (hepatectomy), or in the event of damage to the liver, it can be shown that the fibrinogen values are much below normal. The evidence that prothrombin is formed in the liver—with the help of vitamin K—has already been given (p. 268).

* Evidence is accumulating that the liver is to some extent, at least, a storehouse for protein.

There is evidence, too, that the liver plays a rôle in the manufacture of globin, the protein portion of the hemoglobin molecule. Whipple found liver the most potent among foodstuffs in forming hemoglobin.

We must also bear in mind that aside from the problem of the syntheses of such tissue proteins and others, the problem of the syntheses in the body of certain hormones which are proteins (insulin, several pituitary hormones) and the various enzymes, all of which are probably proteins, must also be investigated. At present our knowledge in this field is little or nothing.

Several suggestive *in vitro* experiments in the field of synthesis, using proteolytic enzymes, will now be recorded.

The experiments *in vitro of* Borsook and Wasteneys on the synthetic formation of "plastein," by using pepsin mixed with concentrated peptic digests, are, at most, suggestive; for the "plastein," showing, to be sure, protein characteristics, is very far from a "native" or "tissue" protein; and the conditions of the experiments, using such high concentrations, are far removed from conditions within the cell.

Wasteneys and Borsook found that a high concentration of substrate and a *p*H of 4 favored synthetic action. Voegtlin, dealing with intracellular proteinases, suggested the following conditions as favoring synthesis of protein within the cell: a relatively high initial concentration of —SH groups (which are needed to activate these intracellular enzymes); and a sufficient concentration of a protein hydrolysate. It is assumed that the enzymes within the cell can bring about synthesis as well as hydrolysis, and that one or the other will take place depending upon the conditions of the medium, as suggested by Voegtlin.

Bergmann (with his collaborators, Fruton and Fraenkel-Conrat) has utilized proteinases for the synthesis of substances of known constitution. When, for example, a 4.2 per cent solution of carbo-

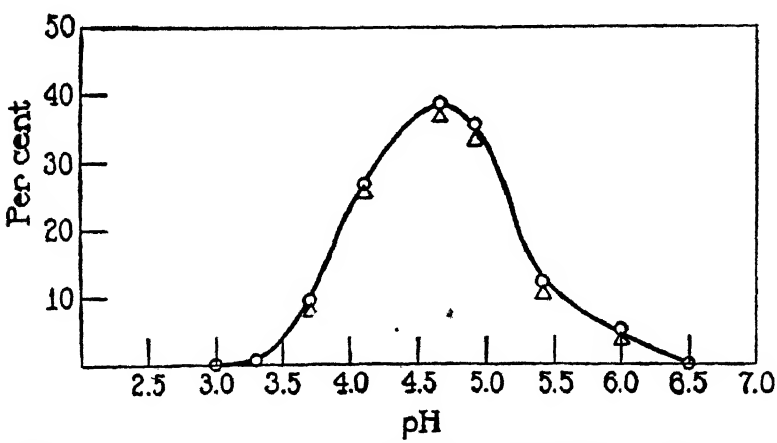
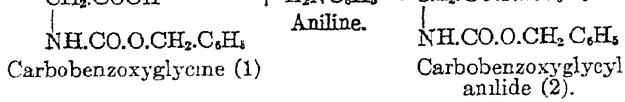


Fig. 90.—*p*H dependence of synthesis of carbobenzoxyglycyl anilide by papain-cysteine. O = amount of anilide isolated after twenty-six hours, expressed in per cent of theoretical maximum; Δ = percentage of anilide formed as determined by titration after twenty-three hours. Concentration of carbobenzoxyglycine, 2.1 per cent; aniline, 1.85 per cent. The *p*H values were determined by means of the glass electrode. (Bergmann and Fraenkel-Conrat.)

benzoxyglycine (1) is incubated with activated papain at 40° C. and *p*H 4.6 in the presence of aniline, carbobenzoxyglycyl anilide (2) is

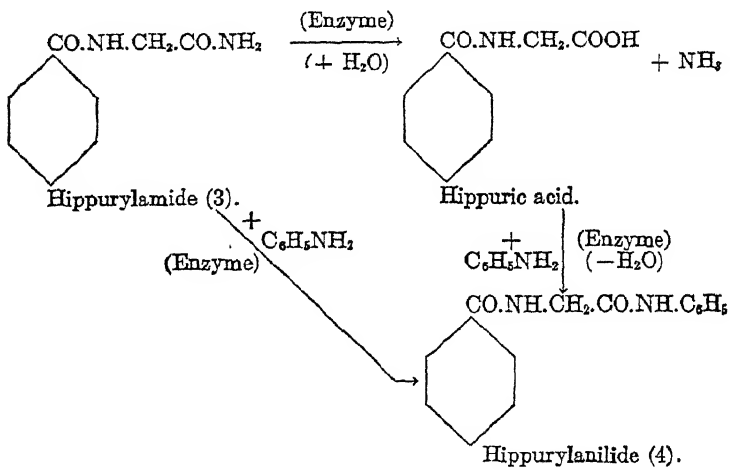


Carbobenzoxyglycine (1)

Carbobenzoxyglycyl anilide (2).

formed. The papain is prepared from the sap of *Carica papaya* (Ceylon), and cysteine (not cystine), glutathione or hydrogen cyanide can be used to activate the enzyme. The optimum *p*H range for the formation of carbobenzoxyglycine anilide is close to 4.6 (Fig. 90).

Whether synthesis or hydrolysis will take place depends upon the nature of the substrate and small structural differences in the latter. Bergmann, Zervas, and Fruton were able to show, for example, that the proteinase which hydrolyzes hippurylamide (3) into hippuric acid and ammonia will, under the same conditions, form hippurylanilide (4) from hippuric acid and aniline. Likewise, hippurylamide and aniline combine, in the presence of activated papain, to form hippurylanilide.



A further step in these synthetic studies was to show that a peptide bond between two amino acid residues could be formed. For example, in the presence of papain, benzoyl-*l*-leucine (1) combines with *l*-leucyl anilide (2) to form benzoyl-*l*-leucyl-*l*-leucyl anilide (3):

$$\underset{(1)}{C_6H_5.CO{-}NH.CH(C_4H_9).COOH} + \underset{(2)}{NH_2.CH(C_4H_9).CO{-}NH.C_6H_5} \rightarrow$$

$$\underset{(3)}{C_6H_5.CO{-}NH.CH(C_4H_9).CO{-}NH.CH(C_4H_9).CO{-}NH.C_6H_5} + H_2O$$

These syntheses can be accomplished not only by papain, but also by bromelin, the proteolytic enzyme of the pineapple, and by cathepsin, obtained from liver. All three enzymes need "activators" (—SH or HCN).

Deamination.—Using the Warburg technic, in which tissue slices and the amino acid under examination are mixed in a Warburg apparatus, enabling one to measure the consumption of oxygen and the liberation of ammonia, it can be shown that both the kidney and the liver are effective deaminizing organs according to the scheme:

$$R\,CH(NH_2).COOH + \tfrac{1}{2}O_2 \rightarrow R\,CO.COOH + NH_3$$

In a number of cases, the keto acids so formed have been isolated. The enzyme responsible for such an oxidative deamination is known as the *amino acid oxidase.*

Two enzymes belonging to the amino acid oxidase class are known: *d*- and *l*-amino acid oxidases. The *l*-form, acting on *l*-amino acids, is physiologically the more important one, since the amino acids obtained from the proteins in foods belong to the *l* series.

Extracts of both the *d*- and *l*-enzymes have been obtained. The *d*-form has even been isolated and shown to be a flavin-adenine dinucleotide joined to a protein (p. 393).

Green has obtained potent extracts of the *l*-form (free from the *d*-variety) from rat kidney and liver, and this one enzyme—presumably just one—catalyzes the oxidation of a number of natural amino acids: leucine, methionine, proline, norleucine, valine, phenylalanine, tryptophan, isoleucine, tyrosine, histidine, cystine and alanine. In all cases the corresponding keto acids and ammonia are formed.

It should be pointed out, however, that there are a number of amino acids upon which this *l*-amino acid oxidase has no action: glycine, dicarboxylic amino acids, diamino acids and β-hydroxyamino acids; which means that in the deamination of these amino acids other amino acid oxidases (or oxidase?) are required.

The enzyme dehydrogenates the amino acid to the corresponding imino acid; the latter hydrolyzes spontaneously into the keto acid and ammonia. The action is reversible. Knoop has succeeded in obtaining the amino acid from the keto acid and ammonia by reduction *in vitro.*

$$\begin{array}{l} R \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \underset{+2H}{\overset{-2H}{\rightleftharpoons}} \begin{array}{l} R \\ | \\ C{=}NH \\ | \\ COOH \end{array} \underset{NH_3}{\overset{+H_2O}{\rightleftharpoons}} \begin{array}{l} R \\ | \\ CO \\ | \\ COOH \end{array}$$

Based on the work of Wieland and Knoop, it is believed that the first step in this deamination process involves the catalytic action of a dehydrogenase, together with a hydrogen acceptor (p. 387). As evidence of this conception, it has been shown that if the two hydrogen atoms of the NH_2 group are replaced by methyl groups, deamination does not take place.

Since the corresponding α-hydroxy acid has, at times, also been obtained, it has been assumed that an alternative series of reactions may be formulated thus:

$$\begin{array}{l} R \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \xrightarrow{H_2O} \begin{array}{l} R \\ | \\ CHOH + NH_3 \\ | \\ COOH \end{array}$$

But many believe that such hydroxy compounds are merely the result of the reduction of the corresponding keto compounds.

There is little loss of potential energy during the deamination process; such losses occur during the further changes of the deaminized portion of the molecule.

All the evidence points to the liver as the seat of formation of urea.

The feeding of isotopic ammonia leads to the elimination of isotopic urea, but no isotopic ammonia. If one now feeds isotopic urea, urea containing the isotope is eliminated, but the ammonia is again isotope-free. The feeding of isotopic amino acids, however, does give rise to the production of isotopic ammonia.

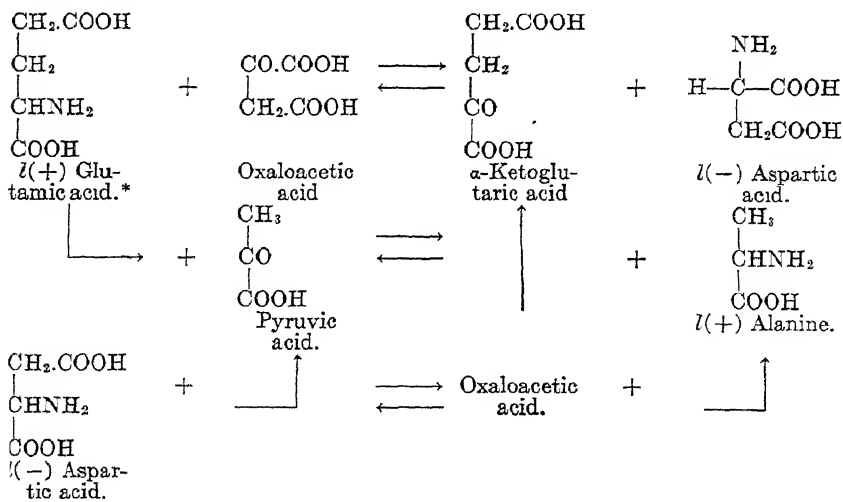
The ammonia, then, is derived from the amino acids and not from urea.

With regard to the fate of keto acid formed as a result of deamination, it has already been indicated that several possibilities are open: ultimate conversion into carbohydrate or fat; reconversion into amino acid; or ultimate oxidation to carbon dioxide and water.

Transamination.—By "transamination" we mean the reaction between an α-amino acid and an α-keto acid, whereby the amino group of the former is transferred to the latter. This reaction is catalyzed by an enzyme, transaminase, present in most tissues, and is reversible:

$$R^1.CHNH_2.COOH + R^2.CO.COOH \rightleftarrows R^1.CO\,COOH + R^2\,CH(NH_2).COOH$$

This type of reaction, using pigeon breast muscle as the source of enzyme, was extensively studied by Braunstein and Kritzmann, and others, with the following substances:



These authors came to the conclusion that the reaction was quite a general one; that any α-amino acid—glycine perhaps excepted—can react with the dicarboxylic acids, α-ketoglutaric and oxaloacetic acids;

* For the significance of *l*(+), see p. 52 (footnote).

and that various α-keto acids, in turn, can react with the dicarboxylic amino acids, glutamic and aspartic acids.

Significant in this connection was the discovery that feeding animals with isotopic amino acids or isotopic ammonia gave rise to a much higher isotopic concentration in the dicarboxylic acids recovered from the tissues than in the other amino acids.*

This generalization, with its wide scope, has been somewhat questioned; but of the importance of transamination in biological reactions there is no doubt; for it helps to explain the synthesis and the degradation of amino acids in animal tissues.

All this leads to the view that the dicarboxylic acids play a very important rôle in the metabolism of protein.

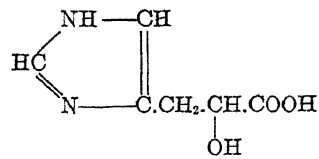
It will be noticed, too, that the three ketonic acids, so important in carbohydrate metabolism, α-ketoglutaric, oxaloacetic and pyruvic (p. 327), become important in protein metabolism because of their property of "transamination." On the other hand, the corresponding amino acids, glutamic, aspartic and alanine, because they can form the ketonic acids, serve in the metabolism of carbohydrates. All this emphasizes connections between protein and carbohydrate metabolism.

The Nitrogen Isotope and Intermediary Protein Metabolism.—Reference has already been made to the use of nitrogen isotope, N^{15}. Schoenheimer and his collaborators (Rittenberg, Foster, Keston, and Ratner) have explored its possibilities in the field of protein metabolism. Several examples of their work will be given. For example, when rats were fed isotopic ammonium citrate there could be recovered from the carcasses, when hydrolyzed, quite a number of amino acids—glycine, glutamic acid, aspartic acid, proline, histidine, lysine, and arginine. Creatine was also isolated. All of these substances with the exception of lysine contained N^{15}. This means that rats can utilize dietary ammonia for amino acid formation.

In another experiment, isotopic *dl*-tyrosine was added to the diet. It was found that about one-half of the isotope given was retained in the tissues, and that practically all the portion assimilated was confined to the tissue proteins. After the proteins found in the liver and carcass were hydrolyzed, tyrosine containing a high concentration of N^{15} was recovered. But what was even more remarkable was to find that various other amino acids which were isolated—arginine, histidine, glutamic acid, aspartic acid—also contained N^{15} (that is N^{15} *in quantities above the normal amounts*). Again, the one exception to the rule was lysine.

In the case of the basic acids, such as arginine and histidine, the question of the distribution of the nitrogen isotope within the molecule was of interest, since these molecules contain more than one nitrogen atom. The arginine was therefore hydrolyzed into ornithine and urea (pp. 357 and 356), and it was then discovered that all the N^{15} resided in the urea and none in the ornithine. Histidine (p. 53), in turn, was converted into imidazole lactic acid,

* Except the amino acid in which the isotope was administered.



Imidazole lactic acid.

This compound contained no N^{15}, from which it was concluded that the nitrogen isotope was here concentrated in the nitrogen of the α-amino group.

The feeding of isotopic tyrosine with the resultant distribution of isotopic nitrogen in the *various* amino acids obtained from tissue proteins suggests both processes involving the opening of peptide linkages and a high chemical activity of tissue proteins.

The experience with these amino acids, including lysine, which behaved abnormally, led to the view that indispensable amino acids may be further divided into two groups: one group, like histidine, contain two metabolically independent chemical groupings: the carbon chain, which is indispensable; and the amino group, which may be derived from some other physiological substance; the other group, represented by lysine, is a single individual chemical unit which has to be supplied as such to the animal.*

Exogenous and Endogenous Metabolism.—Very largely as a result of Folin's work on the output of creatinine—which varies very little despite a varying intake of protein—the idea grew up that the metabolism of protein could be divided into an exogenous and an endogenous variety—the one variable, and not directly concerned with cellular synthesis and analysis, and the other constant, attributed to cellular "wear and tear." The nitrogen excreted by adult animals kept in nitrogen equilibrium (p. 350) was looked upon as representing a variable quantity of dietary nitrogen and a fixed quantity of nitrogen derived from the metabolism of the tissues proper.

Isotopic studies have changed this point of view. Whether we deal with proteins (in the form of amino acids) or fats (in the form of their fatty acids), we find the body constituents involved in continuous chemical processes in which there is a close interaction between the food materials and the body constituents.

To illustrate this point, by feeding animals (maintained in constant weight and in nitrogen equilibrium) isotopic forms of amino acids such as leucine, tyrosine and glycine, only small amounts of isotopic nitrogen could be recovered in the urine, but about one-half was introduced into the body proteins. This could only mean that much of the dietary nitrogen was incorporated by the tissue proteins.

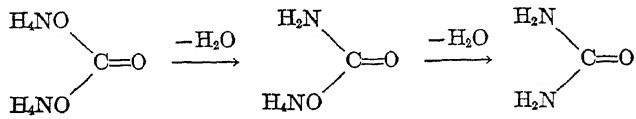
To incorporate the isotopic nitrogen, the peptide linkages of the tissue proteins must open and close continually. "The amino acids . . . mix with others of the same species of whatever source, diet or tissue. By this mixing process they become indistinguishable as to their origin" (Schoenheimer and Rittenberg).

* For some outright speculation, see appendix, p. 571.

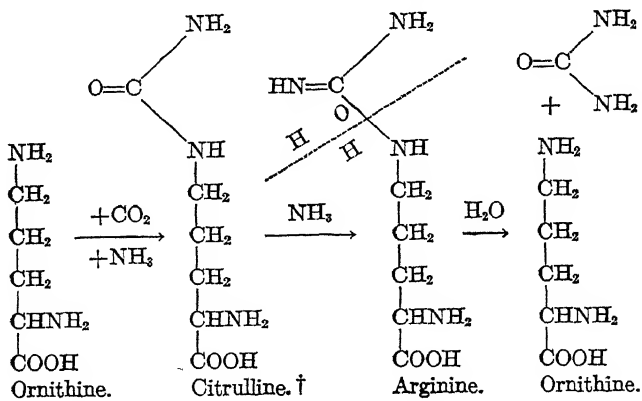
Formation of Urea.—That the liver is intimately involved in the formation of urea can be deduced from the work of Bollmann, Mann and Magath, who showed that in hepatectomized dogs there is a marked decrease of urea in the blood and in the urine.

It has been assumed for a time that urea was formed from ammonia by the combination of the latter with carbon dioxide and water to form ammonium carbonate, and that then, by a loss of water, the carbonate was changed first to carbamate and next to urea:

$$2NH_3 + H_2O + CO_2 \longrightarrow (NH_4)_2CO_3$$



This view was based very largely on perfusion experiments with liver, in which it was shown that both ammonium carbonate and ammonium carbamate can give rise to urea. However, the work of Krebs and Henseleit (using the organ-slice method of Warburg) has shown that the reaction is probably quite different; it involves three amino acids, ornithine, citrulline, and arginine, and the enzyme arginase. According to this scheme, ornithine first combines with ammonia and carbon dioxide to form citrulline; which then combines with another molecule of ammonia to form arginine. In the presence of the enzyme arginase (an enzyme found in the liver), the arginine is decomposed into ornithine and urea. The latter is ultimately eliminated, and the ornithine is available for another cycle of operations.*



What is the evidence for this scheme? Using tissue slices, Krebs found that of all the organs investigated (some 17 in number), only liver had the ability to form urea. The formation of such urea from ammonia is conditioned by the presence of ornithine or citrulline or arginine (which is hydrolyzed to ornithine by arginase). No other

* See appendix, p. 571.

† Archibald finds that human plasma contains 0.3 to 1 mg. of citrulline per 100 cc. This is an important observation, because while ornithine and arginine are among the common amino acids isolated from proteins, citrulline is much less widely distributed.

amino acids or nitrogenous bases can take the place of the three included in the scheme. Furthermore, the liver does contain the enzyme arginase.

That the animal can convert ornithine into arginine is also seen by the fact that when mice are fed ornithine containing deuterium, the arginine, recovered from the body proteins, contains considerable amounts of the isotope.

Evans has shown that the carbon of urea is derived from carbon dioxide by working with tissue slices in the presence of carbon dioxide containing the carbon isotope.

Schoenheimer has further shown that the administration of isotopic ammonia leads to the excretion of isotopic urea, and the arginine isolated from the tissue proteins contains the nitrogen isotope in the amidine or urea portion of the molecule.

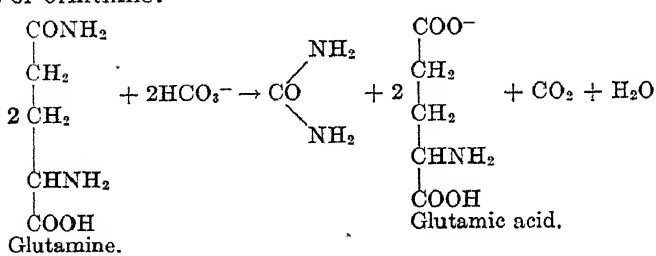
In birds, where we find little urea but much uric acid, the explanation is offered that avian liver does not contain arginase (Table 47).

TABLE 47.—(Baldwin, *Comparative Biochemistry*, Cambridge Univ Press, London.)

	End-product of		Liver arginase.
	Protein metabolism.	Purine metabolism.	
Mammalia . .	Urea	Allantoin[1]	+
Birds. . .	Uric acid	Uric acid	−
Reptilia:			
Snakes, lizards . .	Uric acid	Uric acid	−
Turtles . .	Urea	Allantoin?	+
Amphibia . . .	Urea	Urea	+
Pisces:			
Elasmobranchii (sharks, dogfish, etc.).	Urea	Urea	+
Teleostei (most bony fish)	Ammonia	Urea	+

[1] Uric acid in man, higher apes and Dalmatian dog.

While it is generally agreed that the ornithine cycle is probably the most important mechanism for the formation of urea, other mechanisms cannot be overlooked. For example, it has been claimed, and confirmed, that glutamine and bicarbonate form urea even in the absence of ornithine:



Glutamine. Glutamic acid.

Glutamine—or, at least, a glutamine-like substance—is present in the blood.

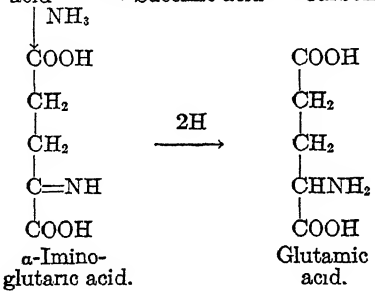
In further support of the glutamine hypothesis is the observation that glutamine will restore urea more rapidly in the liver of starved guinea pigs than will ammonium chloride.

It has also been shown, using liver slices, that the activity of the enzyme arginase can be hindered—by adding large quantities of ornithine—without affecting urea synthesis. Arginase, it will be remembered (p. 357), is an important member in the Krebs cycle.

It is possible that the body utilizes several methods of forming urea; it is equally possible that a complete picture of the scheme is still lacking.

Conversion of Carbohydrate into Protein.—One scheme for the synthesis of protein from carbohydrate has been proposed by Euler and his associates and makes use of the citric acid cycle already discussed under the metabolism of carbohydrates (p. 325):

Carbohydrate → Citric acid → α-Ketoglutaric acid - - - → Succinic acid → Carbohydrate cycle



α-Imino-glutaric acid. Glutamic acid.

The glutamic acid now reacts with keto acids, the latter being transformed into the amino acids (by exchange of NH_2), and the former being converted back again to α-ketoglutaric acid (see also p. 354).

The Conversion of Protein into Carbohydrate and Fat.—The theoretical possibilities here are more or less obvious. If, for the sake of simplicity, we assume the amino acid (representing the protein) to be alanine, then deamination gives rise to pyruvic acid, $CH_3.CO.COOH$, which, we have already seen (Chap. 16), plays its part in the metabolism of carbohydrates. If, furthermore, we accept the possibility of the action of a carboxylase, then pyruvic acid may also be changed to acetaldehyde:

$$CH_3.CO.COOH \xrightarrow{-CO_2} CH_3.CHO$$

and by aldol condensation, the aldehyde may build a fatty acid (Chap. 17).

The actual experimental work has centered itself largely around the use of animals which had been made diabetic by means of a pancreatectomy or, more commonly, by the injection of phloridzin. Such animals excrete glucose. They continue to excrete sugar even when

starved. Since in this state the reserve supply of carbohydrate has practically disappeared we may, perhaps, assume that the glucose formed during such starvation is derived from protein (or its corresponding amino acids).

In the phloridzinized dog, and under fasting conditions, there is a fairly constant relationship between the amounts of nitrogen (representing protein) and glucose excreted. The $\frac{D}{N}$ $\left(\text{or } \frac{\text{dextrose}}{\text{nitrogen}}\right)$ ratio is 3.65, according to Lusk.

If we assume that the percentage of nitrogen in the average protein molecule is 16—which is quite generally done—then we can say that 16 gm. of nitrogen represents 100 gm. of protein. In terms of the dextrose-nitrogen ratio,

$$\frac{D}{N} = \frac{3\,65}{1} = \frac{58}{16}$$

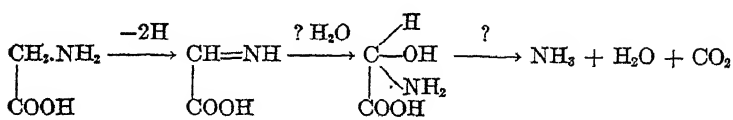
or 100 gm. of tissue protein (16×6.25) under the conditions cited, will give rise to 58 gm. of glucose.

When such fasting, diabetic dogs are fed various amino acids, added quantities of glucose are formed. There are obviously amino acids which can be converted to carbohydrates. Some amino acids, however, give rise to acetoacetic acid. Since this acid is usually regarded as a decomposition product of fatty acids (Chap. 17), the conversion of amino acids into fatty acids (or proteins into fats) may, possibly, be postulated.

The results obtained by Dakin are tabulated in Table 48.

Metabolism of Individual Amino Acids.—It may be assumed that, in many cases, the first step is one of deamination. The oxidation of the residue—if that is what is to take place—is, in reality, the oxidation of some 20 different substances, represented by 20 different amino acids. Each amino acid has to be studied separately. In most instances our knowledge is very meager. We shall attempt to summarize this knowledge in the case of a number of the amino acids which have been studied.

Glycine.*—It is believed, though the evidence is by no means conclusive, that the path of oxidation of this amino acid is via dehydrogenation:



However, to show how uncertain our knowledge is, Bach, working with tissue slices, failed to deaminate glycine, and Green has isolated from the kidney and the liver a glycine oxidase, said to be a flavoprotein, which converts glycine to glyoxylic acid:

* For the formulas of these amino acids, see Chap. 4.

$$\begin{array}{l} CH_2\,COOH \\ | \\ NH_2 \end{array} + \tfrac{1}{2}O_2 \rightarrow \begin{array}{l} CHO \\ | \\ COOH \end{array} + NH_3{}^*$$

This reaction is formulated more in detail as follows:

$$\begin{array}{l} CH_2\,NH_2 \\ | \\ COOH \end{array} \xrightarrow[\text{(enzyme)}]{-2H} \begin{array}{l} CH{=}NH \\ | \\ COOH \end{array} \xrightarrow{+H_2O} \begin{array}{l} CHO + NH_3 \\ | \\ COOH \end{array}$$

Aside from cellular needs for the synthesis of proteins, glycine is used in the formation of bile acids, glutathione, etc.

Under abnormal conditions, glycine may be used by the body for still other purposes. For example, in the diabetic animal the amino acid may form glucose, and in the fasting animal it may be converted to glycogen. The amino acid may also be used for detoxicating purposes (Chap. 11); if benzoic acid is administered, hippuric acid is formed (which represents a condensation of benzoic acid and glycine).

TABLE 48

Substance.	Glucose formation in diabetic animal	Acetoacetic acid formed in perfused liver or diabetic animal.
Glycine	+	−
Alanine	+	−
Serine	+	
Cysteine	+	
Aspartic acid	+	−
Glutamic acid	+	−
Valine	−	−
Leucine	−	+
Isoleucine	−	?
Norleucine		−
Proline	+	−
Oxyproline	+	
Ornithine	+	−
Lysine	−	−
Arginine	+	−
Histidine	+ (?)	− (?)
Phenylalanine	−	+
Tyrosine	−	+

This amino acid has been considered a "dispensable" one in the sense that its absence from a diet otherwise normal does not prevent normal growth in the rat. This view has received very definite confirmation from the more recent work of Rose who, using a mixture of amino acids (but excluding glycine), together with dextrin, sucrose, salt mixture, agar, lard, cod liver oil, and yeast, obtained excellent growth.

It is known that the organism has the ability to synthesize glycine.

* Incidentally, this same enzyme also acts on the methyl derivative of glycine, namely sarcosine, in a similar manner:

$$\begin{array}{l} CH_2.COOH \\ | \\ NHCH_3 \end{array} + \tfrac{1}{2}O_2 \rightarrow \begin{array}{l} CHO \\ | \\ COOH \end{array} + NH_2CH_3$$

It has been shown that rabbits and goats can be fed relatively large quantities of benzoic acid to form hippuric acid; and that the glycine necessary for this condensation is in excess of that which can be derived from the catabolism of the proteins. The amino acid, then, must be synthesized in the body, but we are not certain as to its origin. It is believed that under the abnormal conditions just described—feeding relatively large quantities of benzoic acid—some of the nitrogen which is ordinarily converted into urea and excreted, is diverted from its normal course to build the necessary amino acid.

That glycine is a dispensable amino acid is true in so far as the rat is concerned. However, Almquist has shown that this amino acid is an indispensable one when dealing with the growth of chicks; and, more specifically, with the synthesis of creatine in muscle; for the feeding of glycine invariably results in an increase of muscle creatine.

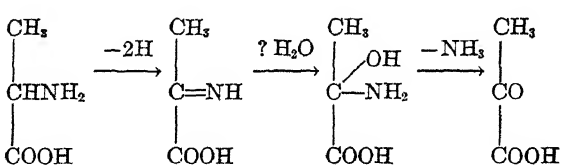
Glycine has been used, with some success, in several cases of muscular dystrophy ("myasthenia gravis"), a form of muscular weakness; but much more work in this direction is needed. (See also under vitamin E, p. 189.)*

The feeding of labeled glycine gives rise to a labeled glutathione which can be isolated from the liver, and to labeled amino acids, isolated from the same organ. However, the introduction of glycine N into glutathione was much faster than into the protein of the same organ. This rapid metabolism of glutathione suggests that the tripeptide may be an intermediate between certain free amino acids and proteins.

The claims that glycine increase the work capacity of human subjects have not been substantiated.

Using isotopic glycine—the stable isotope of carbon in the carboxyl carbon—it has been shown that *some* of the isotopic carbon from the fed glycine may be found in the isolated glycogen from the liver.

Alanine.—The probable path of oxidation is as follows:



What happens once the keto acid is formed—how it is ultimately oxidized to CO_2 and water—is not well known. Of course, this keto acid is important in transamination reactions (p. 354) and in carbohydrate metabolism (p. 324).

* When muscle atrophies, creatine appears in the urine and the amount of creatinine—normally a fairly constant factor—sharply decreases. Neither the injection of creatine nor of creatinine into the sufferer has any effect; both are completely eliminated.

Since glycine aids in the formation of creatine (p. 379), it is believed that this amino acid "influences the muscle so that it can better utilize creatine and thus function more effectively" (Moore).

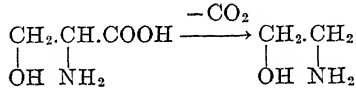
It should be pointed out at this point that, beginning with alanine, we are dealing with amino acids which are optically active. The "natural" amino acids are of the levo (*l*-) variety; and one must be careful to distinguish between the *l*-, the *d*-, and the synthetic varieties; for it does not at all follow that these behave alike in the body.*

Using mixtures of highly purified amino acids instead of proteins, Rose has shown that alanine is a dispensable amino acid. "Rats deprived of this amino acid increase in body weight just as rapidly as do controls receiving a similar ration supplemented with alanine."

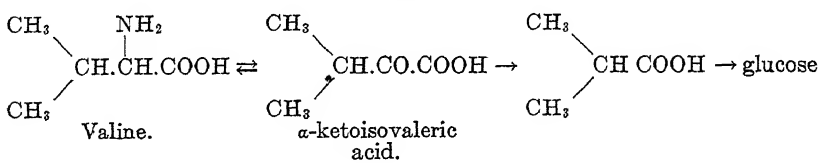
In the diabetic animal, alanine may become a source of glucose.

Serine.—This amino acid is found as a component of some cephalins. Stetten found that some of the dietary serine containing N^{15} is found in the body phosphatids, as well as in the proteins.

It is probable that some ethanolamine may be derived from serine by decarboxylation:



Valine.—That valine is an essential amino acid is known, but our knowledge of its catabolism in the body is very little. Using several forms of valine—*l*(+) valine, *d*(−) valine and *dl*-valine—and α-keto-isovaleric acid in the phlorhizinized dog, Rose has shown that they are converted to glucose. This has suggested to him the following paths:



Leucine.—Of the three closely related amino acids, leucine, isoleucine, and norleucine (see Chap. 4), the first two are indispensable for growth, and the last is probably not essential.

Rose has shown that in the place of leucine and isoleucine the corresponding α-hydroxy and α-keto acids may be used in the diet of rats.

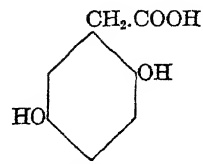
Leucine does not produce glucose in the phloridzinized dog. Added to a perfusing fluid which passes through a surviving liver, large quantities of acetone bodies are formed. This suggests that leucine may undergo a decomposition somewhat similar to fatty acids.

Phenylalanine and Tyrosine.—The experiments of Rose suggest that phenylalanine is an indispensable, and tyrosine a dispensable amino acid. What such experiments probably mean is that, under certain conditions, the former can be transformed into the latter, whereas the reverse process does not take place.

The feeding of phenylalanine containing deuterium results in a deuterium-containing tyrosine, isolated from the liver proteins.

* For the meaning of *l*- and *d*-, see p. 12.

Chemically, the two are so similar that one is tempted to treat them alike. In the rare disease known as "alcaptonuria," the urine blackens on standing. This is due to the oxidation of homogentisic acid:*



Homogentisic acid.
(2,5-Dihydroxyphenylacetic acid)

The administration of tyrosine or phenylalanine to a patient suffering from alcaptonuria increases the quantity of homogentisic acid excreted. This has led to the view that the latter is possibly an "intermediate" compound formed during the course of the oxidation of either one of the two amino acids.

That the degradation in the body of the *d*- and *l*-series of amino acids may take place in different ways is suggested by experiments in which the claim is made that both *l*-phenylalanine and *l*-tyrosine are quantitatively converted into homogentisic acid in the alcaptonuric, whereas only 40–45 per cent of *d*-phenylalanine, 43 per cent of *d*-tyrosine and 68 per cent of *dl*-tyrosine are so transformed.

It has been observed that feeding tyrosine to guinea-pigs on a vitamin C-deficient diet causes the excretion of homogentisic and *p*-hydroxyphenylpyruvic acids. Such excretions can be prevented by the administration of vitamin C (ascorbic acid). However, the administration of ascorbic acid to an alcaptonuric individual seems to have no effect on his excretion of homogentisic acid.

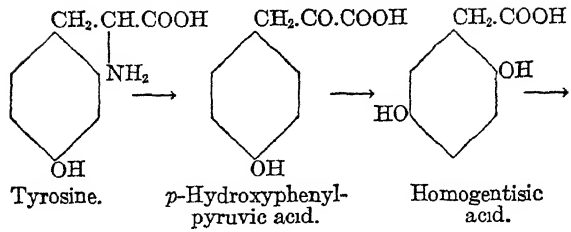
In this connection it is of interest, too, to discover that liver slices from scorbutic guinea pigs fail to oxidize tyrosine, whereas the administration of vitamin C—either *in vivo* or *in vitro*—restores the ability to oxidize. It is, of course, possible that here vitamin C acts as a part of a necessary enzyme system, just as other vitamins have been shown to act.

Lewis has succeeded in isolating homogentisic acid from the urine of rats that were fed phenylalanine over a considerable period of time, often for three or four weeks; and Abbott finds the same to be true when rats are fed a 12 per cent *l*-tyrosine diet.

Both tyrosine (1) and phenylalanine (2) yield acetoacetic acid in perfusion experiments with liver;† so that this four-carbon compound is believed to be formed during the oxidation of (1) or (2). Furthermore, *p*-hydroxyphenylpyruvic acid and homogentisic acid also yield acetoacetic acid in the liver. Some of the steps, then, in the breakdown of tyrosine may be as follows:

* See appendix, p. 571.

† It is also true that in phlorhidzinized animals the two aromatic amino acids give rise to acetone bodies, and yet when the compounds are fed to normal rats, glycogen is formed! (Butts).



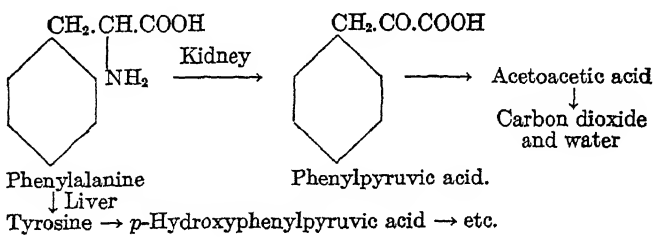
Tyrosine. *p*-Hydroxyphenyl-pyruvic acid. Homogentisic acid.

$$CH_3.CO.CH_2.COOH \longrightarrow CO_2 + H_2O$$

Acetoacetic acid.

In this scheme there are obviously many missing links. For example, the transformation of *p*-hydroxyphenylpyruvic acid into homogentisic acid needs more explanation; nor is it at all clear how the benzene ring is broken to produce acetoacetic acid. Indeed, we are not at all certain that there are not alternate methods of oxidation.

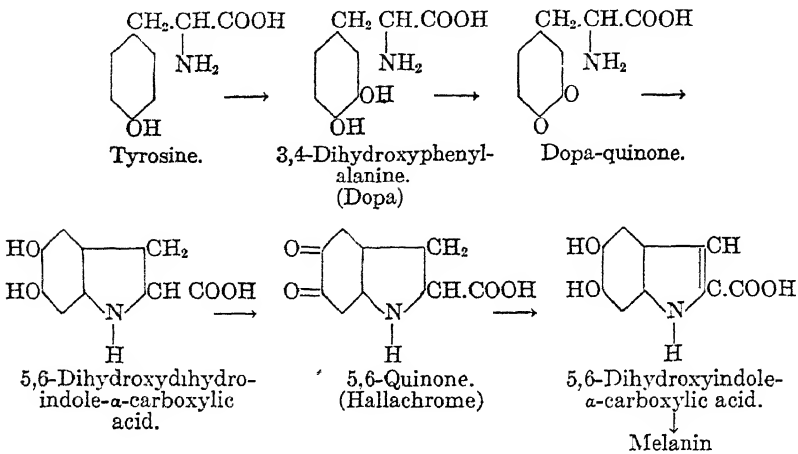
We have already stated that phenylalanine (2) yields acetoacetic acid (3) in the liver. However, phenylpyruvic acid does not yield (3) in the liver, but we do get (3) if kidney slices are substituted. This suggests two alternate paths of oxidation of (2): via phenyl-pyruvic acid in the kidney, and via tyrosine in the liver. That the latter is also probable appears from the work of Medes in connection with a unique case of "tyrosinosis"; here the administration of phenylalanine to the patient produced tyrosine as well as *p*-hydroxyphenylpyruvic acid.



Several workers have described a metabolic abnormality in certain mentally defective patients which is characterized by the excretion of phenylpyruvic acid. This mental condition has been given the name of *phenylpyruvic oligophrenia* (imbecility) and is apparently connected with a disturbance in the metabolism of phenylalanine. In an experiment by Jervis, various amino acids (including tyrosine) were fed to a patient with phenylpyruvic oligophrenia, and phenylalanine alone increased the output of phenylpyruvic acid.

In this connection it may be of interest to note that when rats are kept on a vitamin B_1-deficient diet and fed large amounts of phenylalanine, phenylpyruvic acid is excreted. This does not occur in the presence of vitamin B_1. Since vitamin B_1 is the coenzyme (cocarboxylase) of carboxylase, which acts on compounds of the pyruvic acid type eliminating CO_2, the obvious suggestion is that in this case as well as in that of the mentally defective man described above, the mechanism for the further breakdown of phenylpyruvic acid is absent.

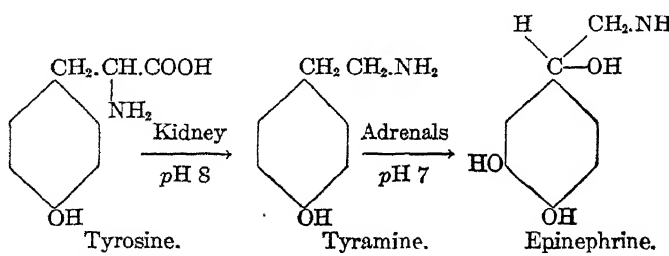
An enzyme tyrosinase converts tyrosine into *melanin*, a brownish black pigment present in skin and hair. The mechanism by which this transformation is accomplished is not known. According to Raper, a hydroxylation of the benzene nucleus possibly takes place, followed by ring closure, whereby indole derivatives are formed.



It is claimed by Ansbacher that *p*-aminobenzoic acid (p. 169) modifies the formation of melanin. In the tyrosine-tyrosinase reaction a typical red stage is reached before the black precipitate of melanin is obtained. In the presence of the acid, the red stage is not seen, and in the place of the black melanin precipitate, a brownish product is obtained.

It has also been claimed that the melanophore* hormone of the pituitary accelerates the tyrosine-tyrosinase reaction.

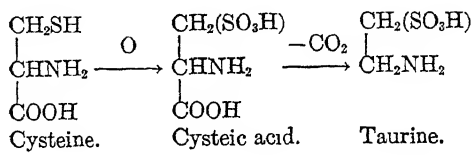
The formulas for tyrosine, epinephrine and thyroxine (p. 479) suggest that tyrosine may be the mother substance of these two hormones. Some evidence for the conversion of tyrosine into epinephrine has been obtained. Using slices of surviving kidney tissue, and with a limited oxygen sypply, tyrosine is decarboxylated and forms tyramine. (In the presence of an excess of oxygen, the tyrosine is deaminized.) The conversion of tyramine into epinephrine, it is believed, takes place in the adrenals, for the latter are unable to form tyramine.



* Dealing with pigment formation.

There is definite evidence that diiodotyrosine (p. 479), found in the thyroid, and undoubtedly derived from tyrosine, is transformed into thyroxine (p. 479).

Cystine, Methionine, and Other Sulfur Compounds.—Cystine is the principal sulfur constituent of the keratin of the skin. It is undoubtedly of importance, not only in cellular growth and repair, but in manufacturing certain constituents of the body. This amino acid gives rise to cysteine and probably to taurine, a constituent of one of the bile acids. It is a constituent of glutathione (p 246) and insulin (p. 314). The taurine is probably formed by a process of oxidation and decarboxylation of cysteine:



Cysteine. Cysteic acid. Taurine.

The sulfur found in the urine as "free" and "combined" sulfate and "neutral" sulfur (Chap. 23) has its origin largely but not exclusively in the cystine of the diet.

It has already been shown (Chap. 11) that cystine can act as a detoxicating agent, combining with bromobenzene, for example, to form a mercapturic acid.

Until the discovery of methionine, it had always been assumed that cystine was an indispensable amino acid. It now appears, from the work of Rose, that whereas methionine can replace cystine in a diet deficient in the latter, the reverse is not true: cystine cannot replace methionine. That methionine can be converted in the body to cystine has been shown by Schmidt, who isolated cystine containing the sulfur isotope S^{35} from the body of animals fed methionine with S^{35}.

Du Vigneaud modified this experiment by feeding rats with methionine containing S^{34} and C^{13} (in the β and γ positions). The recovered cystine showed much S^{34} but no C^{13}, which was a clear indication that in the conversion of methionine to cystine the carbon chain of methionine is not used. But what carbon chain is used—alanine, pyruvic acid . . . ?

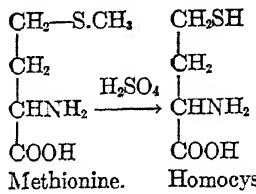
In the rare metabolic disturbance known as "cystinuria," appreciable quantities of cystine as such appear in the urine. A rather remarkable fact is that feeding cystine to such patients does not increase the output of cystine in the urine, but leads to an increased output of sulfates (showing that the amino acid has been oxidized). It has been claimed, however, that feeding proteins rich in sulfur to cystinurics does increase the amount of cystine in the urine; and the suggestion has been made that methionine is involved in this process.

Brand has shown that there is a significant difference in the partition of urinary sulfur when normal and cystinuric types are compared:

	Total sulfate,* per cent.	Neutral sulfur,* per cent.
Normal	95	5
Cystinuric	55	45

As for the intermediate steps in the oxidation of cystine, little beyond what has been indicated is known Perhaps the first step is the conversion into two molecules of cysteine. The fact that a considerable quantity of cysteine is converted into glucose in a phloridzinized dog suggests that one pathway is along lines similar to the three-carbon compounds obtained from glucose.

When methionine is treated with sulfuric acid, demethylation occurs and *homocysteine* is produced.



Methionine. Homocysteine.

Homocysteine is, obviously, the next higher homologue of cysteine. Not only can methionine replace cystine in a diet, but homocystine can also replace cystine. This suggests, according to du Vigneaud, that one of the steps in the intermediary metabolism of methionine is a demethylation process.

By the use of tissue slices of kidney or liver it can be shown that the first stage in the metabolism of methionine is the corresponding keto acid.

The methyl group required to convert homocystine to methionine can be supplied by choline (p. 35) and, to a somewhat less extent, by betaine (p. 50). Du Vigneaud found, for example, that on a diet free of methionine, but containing homocystine, the rat failed to grow; but growth was resumed upon the addition of choline or betaine.

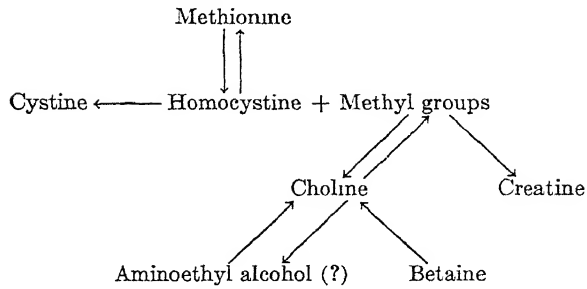
The direct proof of such interrelationships was obtained by the use of compounds containing isotopes. Rats were fed deuterium-containing methionine (with deuterium in the methyl group) on a diet free from methionine and choline. The choline, isolated from the tissues, contained the isotope. Even the creatine, isolated from muscle tissue, contained deuterium.

The reverse is also true: the feeding of deuterocholine and homocystine results in the isolation from the tissues of methionine containing the deuteromethyl group.

The methyl groups of such compounds as choline and methionine act, therefore, as **transmethylating** agents (see, also, p. 379).

The various interrelationships are summarized by Lewis as follows:

* See forms of sulfur in urine (p. 469).



Glutathione, the tripeptide of cysteine, glutamic acid, and glycine, can replace cystine in a diet deficient in this amino acid. This suggests that, during the course of the metabolism of this tripeptide, cysteine (or cystine) may first be liberated.

In discussing cysteine, an optically active acid, it is important to contrast the physiological importance of the *l*- form (which is the naturally occurring form) with the *d*- form. Of course, this applies to all of the amino acids with the exception of glycine; but the work done in this field is somewhat scattered.

In one of his many experiments dealing with sulfur metabolism, du Vigneaud has shown that *d*-cystine cannot support growth in any way comparable to *l*-cystine. This is not always true. For example, both *d*-tryptophan and *d*-methionine can replace the corresponding *l*-forms. If, as a preliminary step to the oxidation of an amino acid, oxidative deamination takes place, whereby the corresponding keto acid is formed, then an explanation for the physiological potency of some *d*- forms might be offered. For it might be assumed that the *d*-form of the amino acid is first transformed to the keto acid and the latter asymmetrically converted into the corresponding *l*-amino acid. In fact, the efficacy of the *d*- form might be brought forward as an argument in favor of the theory of oxidative deamination.

It might be pointed out that feeding *d*-leucine (using isotopic "tracers") enables one to recover *l*-leucine from the proteins of the tissue.

The inability of *d*-cystine to replace *l*-cystine is shown to be due, in part at least, to the much slower oxidation of the former. The feeding of *l*-cystine leads to the conversion of 82 per cent of its sulfur to sulfate (which appears in the urine), whereas only 42 per cent of the *d*-cystine is similarly converted.

Iodoacetic acid interferes with sulfur metabolism. The interference may be due to the following reaction:

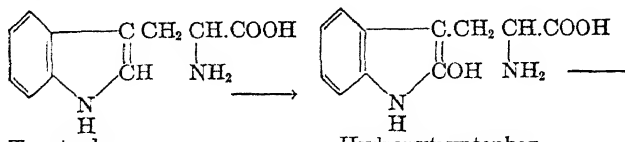
$$R.SH + ICH_2.COOH \rightarrow R.SCH_2.COOH + HI$$

The effect of iodoacetic may be observed by feeding young rats—kept on a basal diet—with it. Growth is very definitely inhibited. However, an extra supply of *l*- cystine (not *d*- cystine), or *dl*- methionine, or *dl*-homocystine results in marked stimulation of growth.

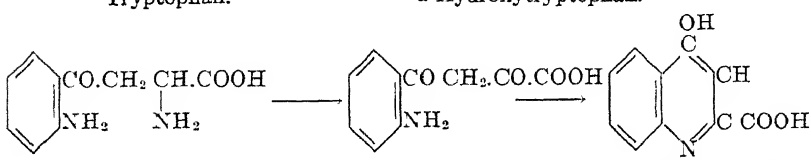
Tryptophan.—This amino acid is one of the so-called "indispensable" acids. From what has already been said, it is not surprising to

find that the corresponding keto acid, indolepyruvic acid, is able to replace trytophan in a tryptophan-deficient diet.

In dogs and rabbits, tryptophan is partly converted to a quinoline derivative known as *kynurenic acid* What appears to be an intermediate product, *kynurenine,* has also been isolated from the urine, as well as by the action of liver slices on tryptophan. The possible pathways of metabolism are here presented:



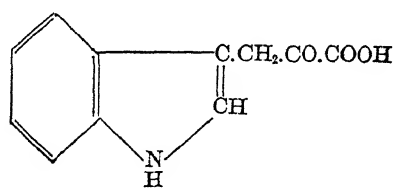
Tryptophan. α-Hydroxytryptophan.



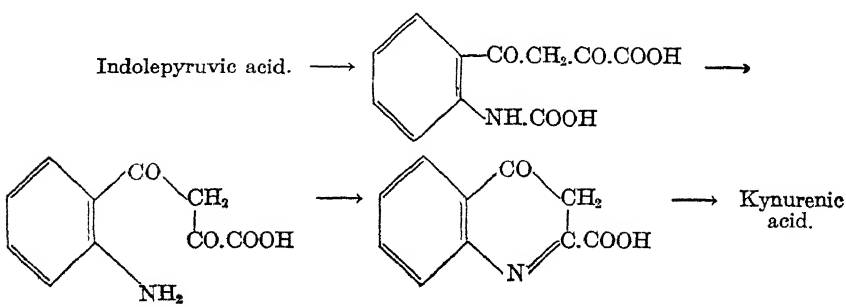
Kynurenine. *o*-Aminobenzoylpyruvic acid. Kynurenic acid.

The stages from kynurenine to kynurenic acid are not clear.

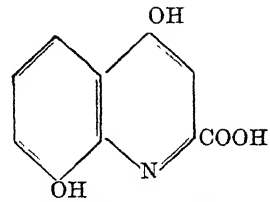
It will be noticed that the outline just presented represents the initial stage of oxidation as an attack on the indole nucleus. There is, of course, the alternate method, whereby the side chain is first oxidized to indolepyruvic acid,



which, ultimately, is partly excreted as kynurenic acid. The course of the reaction may proceed as follows:



An important contribution to the metabolism of tryptophan has been made by Lepkovsky who succeeded in isolating a green pigment from the urine of rats suffering from pyridoxine deficiency (p. 163). This pigment was identified as xanthurenic acid, a quinoline derivative:



Xanthurenic acid.

and the substance disappears from the urine when pyridoxine is added to the diet.

The connection of xanthurenic acid with tryptophan metabolism was suggested by the work of Musajo who found that rabbits and rats excrete xanthurenic acid as well as kynurenic acid on a diet high in protein. He suggested that both these acids had their origin in tryptophan.

This suggestion was confirmed by Lepkovsky who found that on a tryptophan-deficient diet, xanthurenic acid disappears from the urine of pyridoxine-deficient rats and reappears again when tryptophan is added to the diet of such rats.

As showing a species difference—and incidentally adding to the difficulties of interpretation—pyridoxine-deficient dogs, unlike pyridoxine-deficient rats, excrete very little xanthurenic acid.

Kynurenine itself cannot replace tryptophan in the diet, but it can produce kynurenic acid.

Even assuming such schemes as representing the possible metabolic steps in the breakdown of tryptophan, the stages beyond kynurenic acid are unknown.

In contrast to cystine, *d*-tryptophan is as effective a growth stimulant as the *l*-modification; at least, this is true of rats, though the evidence with regard to man is that the *d*-form is much more poorly utilized.

The fact that diets deficient in tryptophan (using the rat) give reduced plasma proteins and hemoglobin values suggests the importance of this amino acid for the syntheses of these proteins.

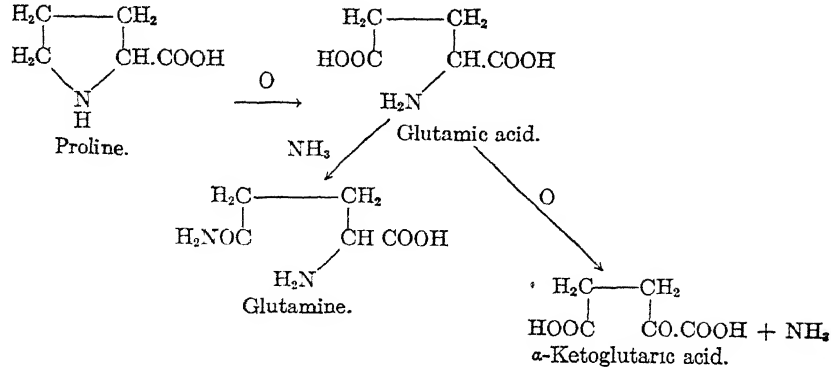
Products such as indole and skatole, the result of the action of bacteria on tryptophan, have already been discussed (Chap. 10).

Proline and Hydroxyproline.—It appears from the work of Rose that proline, and perhaps hydroxyproline, are dispensable amino acids.

The liver is capable of oxidizing these two substances. Proline itself seems to be more rapidly oxidized by the kidney than by the liver. Krebs succeeded in isolating α-ketoglutaric acid and ammonia; which means that glutamic acid (α-aminoglutaric acid) was formed from proline. That glutamic acid is, in all probability, an intermediate product was strengthened by the observation that the addition of proline and ammonium salts gave rise to glutamine.* The scheme pro-

* Kögl has observed that tumor tissue, when hydrolyzed, yields *dl*-glutamic acid, whereas all the other amino acids can be identified as of the *l* (or normal) variety. Analysis of normal tissue belonging to the animal having the tumor tissue showed the glutamic acid to be of the *l* (or normal) variety; this was also true of the other amino acids. This work of Kögl has met with much criticism.

posed, then, is as follows:



Schoenheimer fed isotopic $l(-)$ proline to rats and isolated various amino acids from the carcass and organ proteins. He found deuterium as well as N^{15} in the glutamic acid isolated. The isolated ornithine contained deuterium and N^{15} in the α- and δ-amino groups. Hydroxyproline also showed an appreciable isotopic concentration.

This has led to the following possible scheme (Fig. 91):

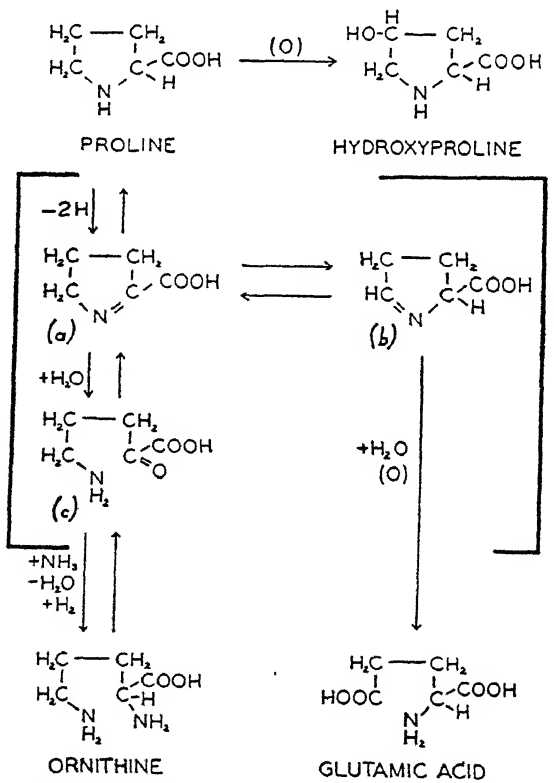


Fig. 91.—Possible metabolic interrelationships of proline, hydroxyproline, glutamic acid, and ornithine. (Hypothetical intermediates are enclosed in brackets.) (Stetten and Schoenheimer, *J. Biol. Chem.*, **153**, 113.)

The first step in the break-down of proline is represented as a dehydrogenation process—similar, in general, to the dehydrogenation of α-amino acids—giving product *a*, which can exist as a tautomer *b*. The hydrolysis and oxidation of *b* yields glutamic acid. Hydrolysis of *a* gives α-keto-δ-aminovaleric acid (*c*), which, under combined reduction and amination, yields ornithine.

Very little is known about the metabolism of hydroxyproline. Lewis finds that whereas the nitrogen of proline is excreted mainly as extra urea nitrogen, only small amounts of extra urea nitrogen are excreted after the administration of hydroxyproline.

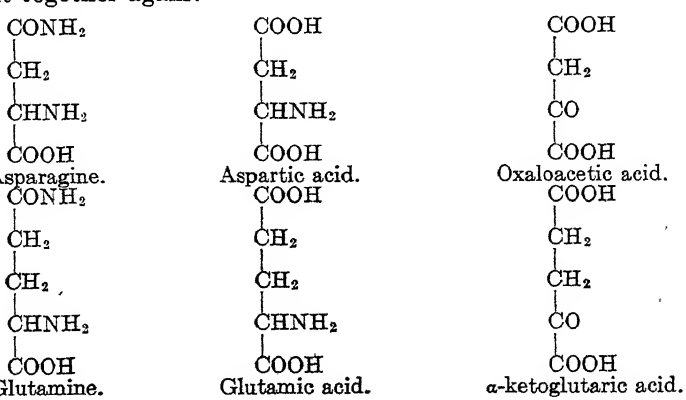
Dicarboxylic Amino Acids.—The three dicarboxylic acids, aspartic, glutamic, and hydroxyglutamic acids, can be removed from the hydrolytic products of casein without affecting the growth-promoting properties of the residue. They are probably "dispensable" amino acids.

With the exception of hydroxyglutamic—about which little is known—aspartic and glutamic have the power of depositing glycogen in the liver, suggesting that the pathway for oxidation is similar to carbohydrates. According to Krebs, glutamic acid is of special importance in the metabolism of nervous tissue. Gray cortex and retina absorb ammonia provided glutamic acid is present. Apparently, glutamine is formed. It is claimed that no other amino acid acts in this way.

It is known that α-ketoglutaric acid (p. 373) is readily converted into glutamic acid *in vivo*. Using radioactive carbon as CO_2, Evans and Slotin have shown that pyruvic acid and carbon dioxide, in the presence of pigeon liver, combine to form α-ketoglutaric acid. Carbon dioxide, then, is used in the synthesis in the body of glutamic acid.

The dicarboxylic amino acids play an important rôle in transamination (p. 354).

These dicarboxylic acids—aside from hydroxyglutamic about which, as we have already said, so little is known—together with their corresponding amides and keto acids, are assuming such importance in protein and carbohydrate metabolism, that their formulas are here brought together again:



Aspartic and glutamic acids give rise to additional glucose when fed to phlorizinized animals.* These same acids administered to rats which

* When subcutaneously injected, phlorizin, a glycoside (p. 14) causes glyco-

have been starved cause a deposition of glycogen in the liver; they are "glycogen formers."

The amides, asparagine and glutamine, occur in nature.

It has already been suggested that glutamine may be a source of urea in the body (p. 358). It is possible that asparagine acts similarly.

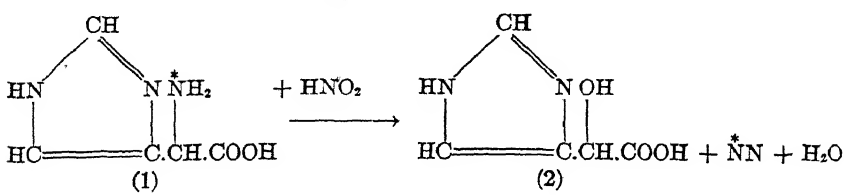
Van Slyke claims that the main portion of the ammonia in the urine is derived from the glutamine of the blood. This apparently settles a problem which had vexed many people.

Histidine.—This amino acid belongs to the list of "essential" amino acids, although the corresponding hydroxy and ketonic acids can replace it in the diet (Harrow and Sherwin). With regard to the oxidation of the imidazole ring itself, it is believed that the enzyme histidase, present in liver, may be involved in such a process. Edlbacher incubated histidine with an aqueous liver extract (from which he later obtained histidase) and isolated ammonia and glutamic acid from the products.

The amine of histidine, histamine, has already been referred to in connection with putrefaction (p. 232). There is, however, an enzyme in the kidney and intestines, histidine decarboxylase, which can also change histidine into histamine, the latter being then stored very largely in the lungs. An enzyme, histaminase, may, under certain conditions, destroy histamine.

It is possible, though by no means proved, that histidine may be connected with purine metabolism.

An insight into the activity of certain groups within the molecule is afforded by the work of Schoenheimer and his associates on isotopic histidine. Histidine containing N^{15} was recovered from the carcass protein of rats fed ammonia containing N^{15}. The isotopic histidine (N^{15}, shown as $\overset{*}{N}$) (1) was converted to its imidazolelactic acid (2) by means of nitrous acid. The derivative so formed contained normal nitrogen; and therefore the isotope in the histidine must have resided in the α-amino group:



"Histidine," say the authors, "is subject to a continuous process of successive deamination and amination involving only the α-amino group."

There is some evidence to show that in pregnancy a disturbance in the metabolism of histidine may develop.

The feeding of *l*(−) histidine gives rise—after eight hours, though —to a deposition of glycogen in the liver.

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suria. This form of diabetes is called "phlorizin diabetes," because it prevents the kidneys from reabsorbing glucose.

Three histidine compounds found in the body—ergothioneine (p. 53), which is found in the blood, carnosine, a dipeptide of histidine and β-alanine, and anserine, a methyl derivative of anserine (see p. 447) are found in muscle.

Arginine.—As to its metabolism, very little is known. It probably first breaks down to ornithine and urea, but what becomes of the ornithine (apart from its use in urea formation) is not known. Ornithine may form glycogen when administered, and this suggests a pathway similar to the carbohydrates.

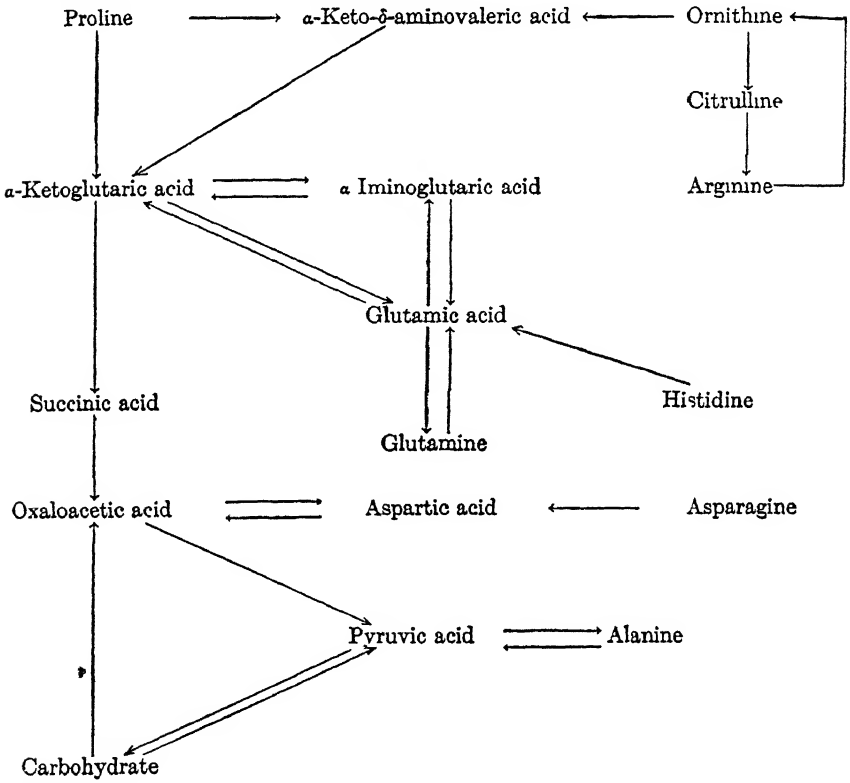


Fig. 92.—Probable interrelationships between a number of amino acids and other compounds. (Lewis and Garner, *Ann. Rev. Biochem.*, 9, 286.)

Rose placed arginine among the indispensable amino acids, but with certain reservations: he found that arginine can be synthesized by the animal organism, but not at a sufficiently rapid rate to meet the demands of *normal* growth. The exclusion of arginine from the diet caused a decrease in the growth rate to about three-fourths of the normal value.

This work by Rose was with rats. Elvehjem, Almquist, and their co-workers, using the chick, found that arginine is necessary for rapid growth. Apparently, the chick is not able to synthesize sufficient arginine to maintain body weight. Neither ornithine nor ornithine plus urea were able to replace arginine in the diet.

The probable interrelationships between several amino acids, suggested by work with tissues and tissue extracts, are graphically illustrated by Lewis and Garner in Fig. 92.

Lysine.—Little is known about this amino acid. When isotopic amino acids are fed to animals, the various amino acids isolated from the tissue proteins contain the isotope; lysine is the one exception. In contradistinction to other amino acids, once lysine is deaminated, it cannot be aminated again to form the amino acid.

This behavior of lysine requires further examination. In many cases, when the unnatural or *d*-form of the amino acid is fed, the body has the ability to convert the *d*- into the *l*-form by a process of deamination and reamination, the original nitrogen being replaced. This statement may be put in another form: the ability of the body to utilize the *d*-form of an amino acid probably depends upon its ability to convert the *d*- into the *l*-form.

When, for example, *d*-leucine, containing N^{15} in the amino group and deuterium in the carbon chain, is fed and the natural *l*- leucine is isolated from the tissues, the natural form contains deuterium but no N^{15}. During inversion in the body, deamination and reamination had taken place, and the original nitrogen had been replaced.

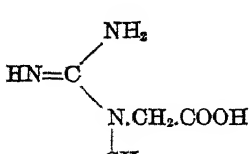
If, now, we feed *d*-lysine, containing N^{15} in the α-amino group and deuterium in the carbon chain, and recover the natural *l*-lysine from the tissues, both deuterium and N^{15} are absent. The body (the rat in this experiment) is unable to convert the *d*-lysine into its natural form.

It is known that *d*-lysine, unlike most of the other *d*-amino acids, will not support growth. The explanation now given is that in the case of lysine, when the *d*-form is fed, the preliminary deamination, giving rise to the keto acid, is *not* followed by the reamination. With other amino acids (*d*-leucine is a partial exception) the *d*-forms in the body are, to some extent at least, first converted to the keto acids and then reaminated to the natural (*l*-) amino acids.

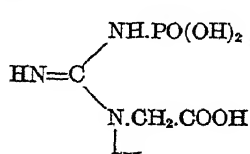
In accordance with what has already been said, we find that lysine is not deaminized by kidney or liver slices—a reaction, once again, in striking contrast to most of the other amino acids.

CREATINE AND CREATININE

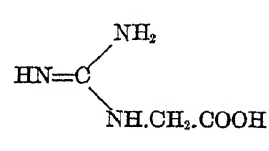
Creatine, either free or in the form of phosphocreatine, is found in muscle, brain, and blood; creatinine, on the other hand, is a constituent of blood and of urine. Creatine appears to be confined to vertebrates. In invertebrate muscle, arginine takes the place of creatine. The structure of these compounds suggests a relationship to a number of amino acids and nitrogenous substances.



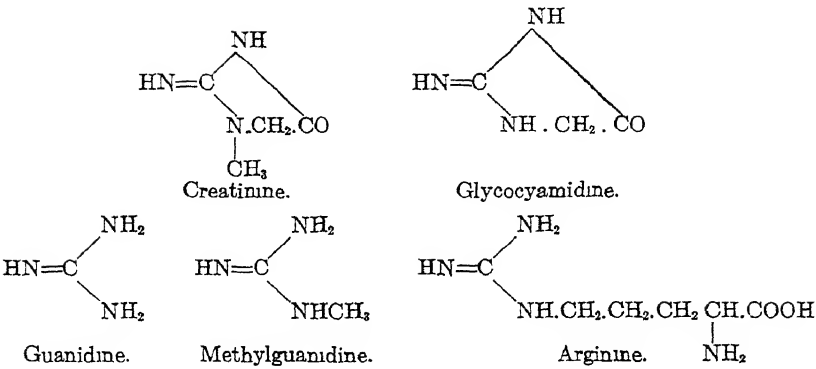
Creatine.



Phosphocreatine.



Glycocyamine.



Creatine and creatinine can be very easily transformed into one another *in vitro*. The creatinine is a much more powerful base than creatine. Treatment of the latter with acid converts it to creatinine. In an alkaline medium, a partial reversal of the process takes place, an equilibrium point being finally reached. In N/2 HCl, at a temperature of 117° C., the change from creatine to creatinine is practically complete in fifteen minutes.

One general method of quantitatively estimating these substances is based on a colorimetric procedure: the red color obtained when creatinine and sodium picrate are mixed. By first converting creatine to creatinine (with acid), the former substance can also be determined by this method.

TABLE 49.—TRUE CREATINE AND CREATININE CONTENTS OF RAT TISSUES. (Baker and Miller, *J. Biol. Chem.*, **130**, 396.)

	Creatine.	Creatinine	Ratio, creatine to creatinine.
	mg. per 100 mg.	*mg. per 100 mg.*	
Gastrocnemius	534	4 8	111
Diaphragm	424	3 2	133
Heart muscle	192	3 0	64
Testis	297	4.0	74
Brain	129	1 0	129
Kidney	26	0 7	36
Spleen	15	0 3	50
Lung	14	0 1	140
Liver	6	0 1	60

Another and very ingenious method we owe to Miller and Dubos. They isolated soil bacteria which could adapt themselves to grow on creatinine as their sole source of carbon and nitrogen. An enzyme formed by these bacteria is highly specific: it acts only on creatinine and not even on closely related compounds which also give color reactions with alkaline picrates and which are also present in the blood.

In using this method, creatine is converted to creatinine and esti-

mated by the use of alkaline picrate before and after incubation with the enzyme.

The results are given in table 49.

The Origin of Creatine.—When isotopic glycocyamine is fed to rats, both isotopic creatine and creatinine are formed. Creatinine is formed in two steps: (a) the production of glycocyamine; and (b) the methylation of the compound produced.

As to the origin of glycocyamine, Fig. 93 shows the effect of feeding glycine and arginine on the excretion of glycocyamine:

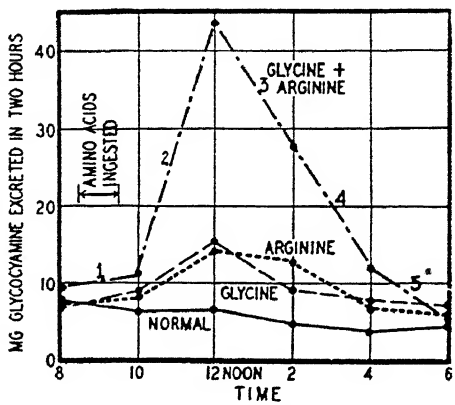
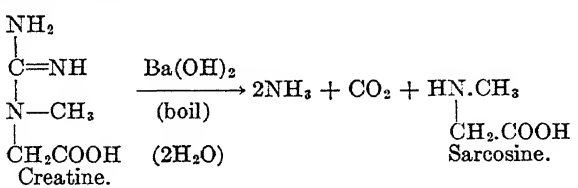


Fig. 93.—Glycocyamine excretion in urine. The ordinates represent the glycocyamine excreted in the urine in the two-hour period ending at the time indicated, the abscissae, the time at which the urine was voided. The amino acids were ingested, on each of the four days, in four portions between 8:30 and 9:30 A.M. (Borsook, Dubnoff, Lilly and Marriott, *J. Biol. Chem.*, **138**, 406.)

Borsook, working with liver slices, showed that, to be sure, glycocyamine is converted into creatine, but at a very slow pace. However, the addition of methionine, supplying additional methyl groups, accelerated the process a great deal.

Using isotopic compounds, Bloch and Schoenheimer found that among various compounds used, arginine and glycine were the most effective creatine formers. By degrading the creatine formed, the particular nitrogen supplied to creatine could be located:

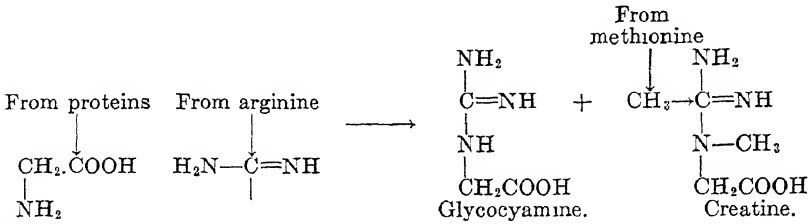


All of the N in the amidine group of creatine is recovered as NH_3, and the remaining N is recovered in the form of sarcosine.

When isotopic glycine was fed, the isotope was located in the sarcosine fraction of creatine. On the other hand, the feeding of isotopic arginine concentrated the isotope in the ammonia fraction obtained from creatine.

In this way it could be shown that the amidine group in creatine is derived from arginine, and the sarcosine portion, from glycine.

The origin, then, of creatine, can be summarized as follows:



While the methyl group of methionine is used for the building of the creatine of muscle, neither creatine, nor creatinine, nor sarcosine can supply its methyl group to convert homocystine to methionine.

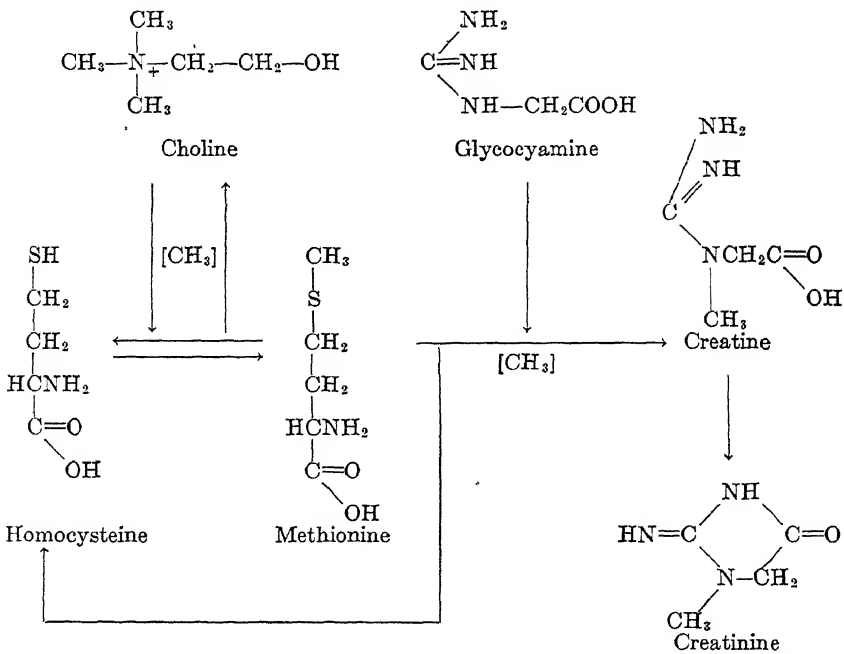


Fig. 94.—Scheme of the metabolic interrelationships of methionine, choline, and creatine. (Du Vigneaud, *Harvey Lectures* (1942–1943), p. 55.)

The Relation of Creatine to Creatinine.—Folin was the first to show that the amount of creatinine excreted in the urine of any normal individual is remarkably constant. But what is its origin? It has been rather generally accepted that the mother substance is the creatine of muscle. Benedict and Osterberg showed, for example, that feeding creatine to dogs over a comparatively long period—from five to ten weeks—gradually increased the creatinine output. The slowness with which this apparent transformation is accomplished suggested to the authors that, in the place of a direct transformation, there are a number of intermediate steps involved.

This old problem has now been definitely settled. Feeding isotopic creatine gives rise to isotopic creatinine in the urine.

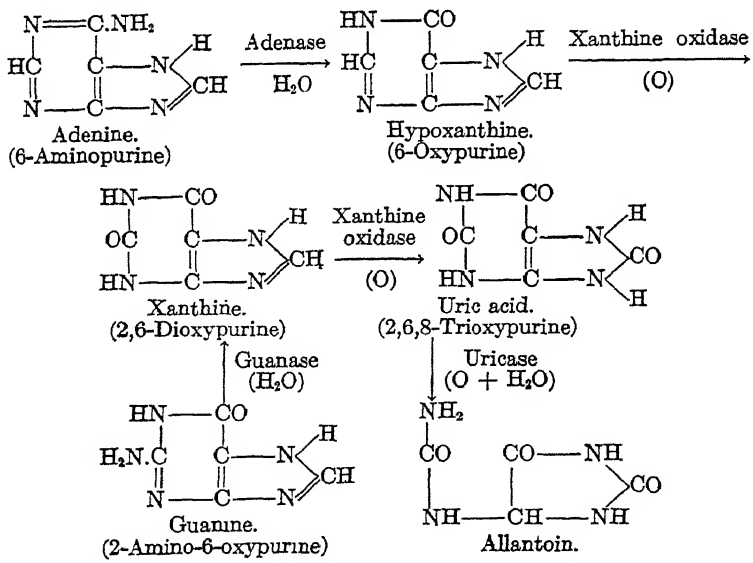
Even more striking, however, is it to feed isotopic creatine for some time and then to follow this feeding with a creatine-free diet. At this later stage, the urinary creatinine has the same isotopic content as the body creatine.

While it is possible, *in vitro*, to convert creatine into creatinine, and creatinine back again to creatine, *in vivo* the process is an irreversible one. The feeding of isotopic creatinine does not result in the formation of isotopic creatine, but in the elimination of unchanged creatinine.

Figure 94 emphasizes interrelationships which have already been discussed in this section and elsewhere.

PURINE AND PYRIMIDINE METABOLISM*

The purines (and pyrimidines) result from the hydrolysis of nucleic acid. In the body some of them are changed to uric acid. This is the "end-product" of purine metabolism in man, the uric acid being eliminated as such. In many mammals, however, the uric acid is further oxidized to allantoin.† These various changes are brought about by specific enzymes which seem to be quite widely distributed, although they are found in abundance in the liver. The changes can be represented as follows:



The xanthine oxidase, involved in two of these reactions, has been studied by Ball, who comes to the conclusion that it is probably a flavoprotein.‡

* The student would do well to refer to Chap. 5 again.

† Allantoin is found in the excretion of maggots. It has been known for a long time that when certain wounds are infested with live maggots, healing is often rapid. It is now believed that the curative substance is none other than allantoin itself.

‡ Ball also believes, incidentally, that the enzyme responsible for the Schar-

Not only is the body able to catabolize purines, but it is also able to build nuclear material. The classical example is that of the salmon which builds nuclein material during its long passage to its spawning destination during a period when it does not eat.

The seat of much purine metabolism is believed to be the liver. Man and the higher primates form uric acid as a result of the ultimate oxidation of purines. In other mammalia (except the Dalmatian dog), the uric acid is further oxidized to allantoin. In birds and in reptiles, uric acid is the main product of nitrogenous metabolism (comparable to urea in man). The lack of arginase in the liver of the bird has been offered as an explanation for the absence of urea. In any case, the liver of the bird does not form urea.

The important pyrimidine bases obtained from nucleic acid are uracil, thymine, and cytosine (see p. 85).

Some bits of information regarding purine and pyrimidine metabolism have been obtained by using the now classical tool: isotopes.

For example, Hevesy, using radioactive phosphorus (P^{32}) in the form of phosphate, has examined the liver fractions after injections and finds that the "turnover" of phosphorus in the nucleic acid portion is less than in the phospholipid or protein portion.

Using pigeons, and studying the problem with the aid of the nitrogen isotope (N^{15}), Schoenheimer found that ammonia nitrogen was incorporated in the uric acid which was excreted and also—but to a smaller extent—in the mixed purines of the internal organs. With N^{15} urea in the place of N^{15} ammonia, the results were generally negative.

Rats gave results similar to those obtained with birds. Here allantoin is the end product of purine metabolism, and this substance showed the isotope.

NITROGENEOUS CONSTITUENTS IN BLOOD

Clinically, these nitrogenous blood constituents often assume importance, for they may reveal a picture of abnormal disturbances in the metabolism of proteins.

Table 50 gives first some normal figures of nitrogenous substance in blood (the three last figures pertaining to sulfur are also pertinent, since such sulfur compounds originate in proteins):

We have already discussed the proteins of the blood (p. 265) and shall now turn our attention to the nonprotein nitrogen (NPN), representing the compounds below fibrinogen in Fig. 50. Just a few will be mentioned.

Increased urea nitrogen occurs in chronic nephritis. In terminal

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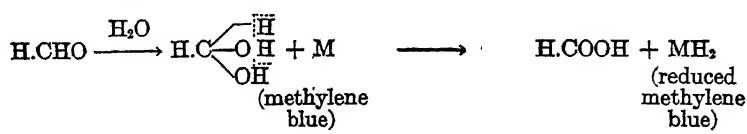
dinger reaction of milk is none other than xanthine oxidase. In this Schardinger reaction, the reduction of methylene blue by aldehyde is rapidly catalyzed by fresh milk and much more slowly by milk which has been heated:



stages, values as high as 200 mg. (per 100 cc. blood) have been obtained. High values are also shown in acute intestinal obstruction, bichloride of mercury poisoning, cardiac failure, etc.

TABLE 50.—PHYSIOLOGICAL NORMALS OF NITROGEN METABOLISM. [Compare with p. 256. Proteins and Amino Acids (1944), p. 48.]

	Blood (per 100 cc.)
Plasma Proteins	6 0–7 2 gm
Albumin	4.6 ± gm.
Globulin	2 0 ± gm.
Fibrinogen (plasma)	0 3–0 6 gm
Non-protein Nitrogen	25–35 mg.
Urea Nitrogen	10–15 mg.
Uric Acid	2–3 5 mg.
Ammonia Nitrogen	0.1–0 2 mg.
Undetermined Nitrogen	4–18 mg.
Amino Acid Nitrogen	4–4 5 mg.
Creatinine	1–2 mg
Creatine	3–7 mg
Inorganic Sulfur*	0.1–1 1 mg.
Ethereal Sulfur*	0 1–1 0 mg
Neutral Sulfur*	2 2–4 5 mg

* For their significance, see pp. 469, 470

Values of urea below normal are observed in hepatic insufficiency—values as low as 5–10 mg. have been reported in acute yellow atrophy of the liver (urea synthesis is impaired). Subnormal amounts also occur during pregnancy: the urea nitrogen may be as little as 6 mg. and the NPN (of which the urea is so large a part) may be 20–25 mg.

Increased blood uric acid is observed in nephritis (possibly 4–10 mg.); gout (which is connected with a disturbance of purine metabolism), giving figures during attacks of as high as 6–10 mg.; leukemia (a disease connected with an increase in the number of leucocytes in the blood); pernicious anemia; urinary obstruction; eclampsia (convulsive attacks); intestinal obstruction; impaired liver function; etc.

Decreases in uric acid below normal amounts are not common.

Increases in creatinine may occur whenever blood urea is increased. In chronic nephritis, figures as high as 35 mg. have been observed.

Amino acids may show a substantial increase in hepatic insufficiency; here the function of the liver in forming urea has been impaired; which also means that urea values will show subnormal amounts. As much as 200 mg. of amino acids (per 100 cc. of blood) have been observed in acute yellow atrophy of the liver.

The total nonprotein nitrogen (urea, uric acid, amino acids, creative, creatinine, etc.) will usually show increases in those instances where blood urea increases. In chronic nephritis, for example, values as high as 400 mg. have been observed.

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CHAPTER 19

BIOLOGICAL OXIDATIONS

In this chapter we will consider the mechanism by which the molecular oxygen, brought to the cell, is able to oxidize substances in the cell ("metabolites").

That molecular oxygen by itself is incapable of such oxidations has been proved in many experiments. For example—to take a very simple case—hypoxanthine in contact with an extract of liver is easily oxidized to xanthine in the presence of ordinary oxygen; yet *in vitro*, molecular oxygen has no such effect. In fact, hypoxanthine can be boiled with nitric acid without any appreciable change. The liver contains some catalyst, some enzyme (which is called xanthine oxidase) which catalyzes the reaction.

That tissues contain such "oxidases" can be readily shown in the very simple experiment of treating a solution of guaiac with an aqueous extract of the potato. The guaiac contains a phenolic derivative which, when oxidized, changes to a blue color (guaiac blue). The blue color is very readily obtained when the tissue extract and the guaiac solution are mixed in the presence of oxygen.*

The mere knowledge that there are enzymes—to which the general name "oxidases" has been given—which catalyze oxidative reactions within the cell brings with it more questions than answers. Are we dealing with one oxidase or with many oxidases? What is their chemical composition? What is the mechanism involved in their reaction with metabolites?

We shall attempt to answer these questions in so far as they can be answered today.

The Activation of Oxygen.—One of the earliest theories dealing with biological oxidation involved a catalyst activating "molecular" into "active" or "atomic" oxygen, the latter being then in a position to attack the metabolites within the cell. This theory emphasizes the importance of "activating" oxygen, but discards the necessity of "activating" the metabolite.

In 1927, Warburg postulated such an oxygen "activation" based on his discovery of the "respiratory enzyme," a heme compound, present in all cells, similar to but not identical with hemoglobin. It is the iron in the enzyme, according to Warburg, which is primarily responsible for the reaction, being first oxidized and then reduced:

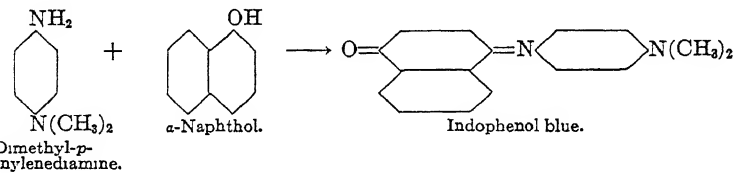
$$\underset{\text{("Respiratory enzyme")}}{\text{X.Fe}} + \text{O}_2 \rightleftarrows \text{X.FeO}_2$$

$$\text{X.FeO}_2 + 2\text{A} \rightleftarrows \underset{\text{(Organic molecules)}}{\text{X.Fe}} + 2\text{AO}$$

* Catechol (p. 399) is very often used in place of guaiac. With the "oxidase" the catechol goes through a series of color changes: green, yellow, brown, black.

From the work of Keilin and others, it now appears that this "respiratory enzyme" is identical with an "indophenol oxidase" or "cytochrome oxidase" which is important in the activity of cytochrome (p. 395). What, for a time, was hailed as the oxidizing agent responsible for biological oxidations now turns out to be but one of a number of substances.

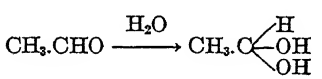
The "indophenol oxidase," present in various animal and some vegetable tissues, gets its name from the fact that when mixed with dimethyl-*p*-phenylenediamine and α-naphthol in the presence of oxygen, an indophenol, a blue substance, is produced; and this reaction is used as a method of identifying the enzyme.



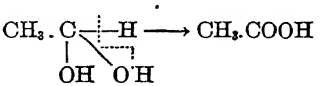
Dimethyl-*p*-phenylenediamine. α-Naphthol. Indophenol blue.

The Activation of Hydrogen.—The next advance we owe to Wieland. His work has played a major rôle in our understanding of biological oxidations. According to Wieland, in the presence of the suitable enzyme (now commonly called a *dehydrogenase*), certain hydrogen atoms in the metabolite are made "active" and removed. In order that the reaction may continue, the hydrogen liberated has to be removed continuously. In the simplest examples, the "hydrogen acceptor" can be oxygen itself. What, however, is more often the case, the hydrogen is passed on to one or more "carriers" before it is finally oxidized.

Wieland was led to this "dehydrogenase" theory by experiments involving finely divided platinum or palladium black. He found, for example, that the addition of palladium black to a solution of acetaldehyde converted the latter to acetic acid. What happens, probably, is that the aldehyde first combines with water to form an intermediate hydrate



and that then 2 atoms of hydrogen are removed from the water by the palladium:



In the presence of a "hydrogen acceptor," such as quinone, or reducible dyes, such as indigo and methylene blue, the hydrogen, temporarily absorbed by the metal, is transferred to the "acceptor," and the reaction proceeds to completion. The two reactions are:

$$AH_2 + Pd \longrightarrow A + PdH_2$$

Metabolite.

$$PdH_2 + B \longrightarrow Pd + BH_2$$

(Quinone)

The Activation of Hydrogen and Oxygen.—The discovery of cytochrome by Keilin (p. 394), which has to do with oxygen "activation," led to the view that biological oxidation is possible only if both the hydrogen of the metabolite and the oxygen are activated. This is probably true in many cases. But even here, the more general application of such a theory is possible if we consider that "carriers" are also essential to the system; in other words, before the hydrogen can be transferred to the oxygen, it must be "carried" by one or more substances. The most substantial progress recently has been made in determining the nature of some of these "carriers."

"Hydrogen Carriers."—A "hydrogen carrier" is a substance present in the cell which can "accept" hydrogen and so be reduced, and can then be oxidized again (by transferring the hydrogen to another "carrier" or by oxidation of the hydrogen by oxygen). The "carrier," then, is the "hydrogen acceptor"; but we must point out that when we talk of "carriers" we have in mind substances found in the cell and not palladium black or hydroquinone.

These "carriers" include pyridine nucleotides, flavoproteins, cytochromes, etc.

An Outline of the Theory Underlying Biological Oxidations.—We shall first give a summary of the process and then discuss it in some detail. The outline may be given as follows:*

$$\left.\begin{matrix}\text{Metabolite}\\ \text{or}\\ \text{substrate}\end{matrix}\right\} \xrightarrow[\text{(dehydrogenase)}]{-2H} \text{pyridine nucleotides} \longrightarrow \text{flavoproteins} \longrightarrow$$

(?) succinate — fumarate cycle ⟶ cytochromes† ⟶ cytochrome oxidase ⟶ oxygen.‡

At one end of the cycle hydrogen is removed from the metabolite (or substrate, or, as it may also be called, foodstuff), and this hydrogen is carried by a series of "carriers" until it ultimately combines with oxygen to form water.

In the presence of an enzyme, dehydrogenase, hydrogen atoms in the substrate are "activated" so that they can be removed and turned over to an acceptor or "carrier."§

In the presence of dehydrogenase (an enzyme with the characteristics of a protein), and in the presence of a "carrier"—also called a "coenzyme"—the substrate loses two hydrogen atoms; or better still, two electrons and two hydrogen ions. At this stage the "coenzyme" is usually one of the pyridine nucleotides (p. 391), and it receives two electrons and one hydrogen ion, the other hydrogen ion remaining in the environment.

* Not all biological oxidations go through these various steps. See p. 390.

† Some believe that the order is cytochrome *b* → cytochrome *c* → cytochrome *a*.

‡ Probably a more correct way to visualize this system is to assume that not hydrogen but electrons $\left(\underset{2H \rightarrow 2H^+}{^{-2e}}\right)$ are carried by the "carriers"—which are alternately oxidized and reduced—and that the H^+ present in the environment finally joins with oxygen $\left(\underset{O \rightarrow O^-}{^{+2e}}\right)$ to form water.

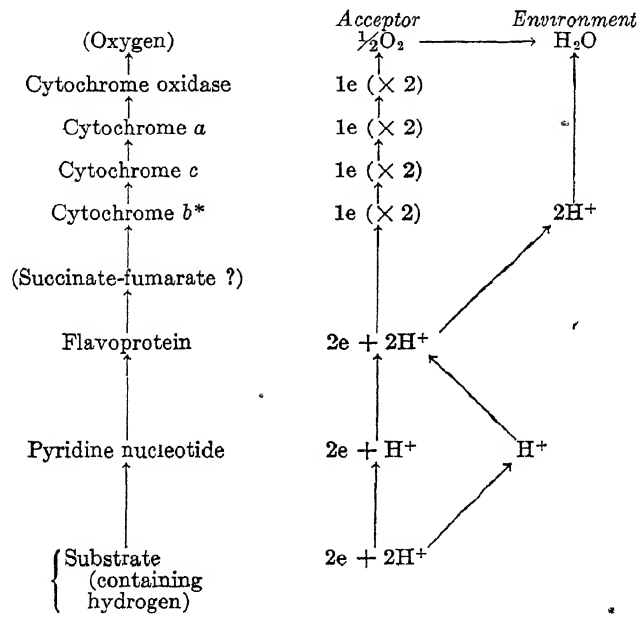
§ It is believed that each substrate requires a specific dehydrogenase.

The result of this reaction is that the substrate is oxidized and the pyridine nucleotide is reduced. The latter is reoxidized by reacting with a flavoprotein (p. 392). Two electrons and one hydrogen ion from the nucleotide, and one hydrogen ion from the environment, are transferred to the flavin of the flavoprotein.

The flavoprotein having been reduced in its turn, must be reoxidized. According to Szent-Gyorgyi, this is done through the mediation of the succinate-fumarate system. Others believe that the flavoprotein reacts directly with cytochrome *C*.

In either case, the reaction proceeds in its stepwise direction, with its alternate reduction and oxidation, until the terminal stage, in the shape of oxygen, is reached.

Using the outline suggested by Ball, we can again summarize such reactions as follows:



The cytochrome system needs further elaboration. Oxygen combines with cytochrome oxidase† (p. 395) in the reduced form to form $Fe^{++}O_2$.cytochrome oxidase.‡ In the presence of H^+, the oxygen removes an electron from the Fe^{++}, oxidizing it to Fe^{+++}, and combines with H^+ to form water:

* "Although several of the known flavoproteins react with reduced triphosphopyridine nucleotide, none of them reacts directly with cytochrome *c*. Cytochrome *b* and hydrogen carriers, presumably acting between cytochrome *c* and the flavoproteins, have therefore been postulated as part of this scheme, but no concrete evidence to support such postulated mechanisms has been presented" (Haas, Horecker and Hogness).

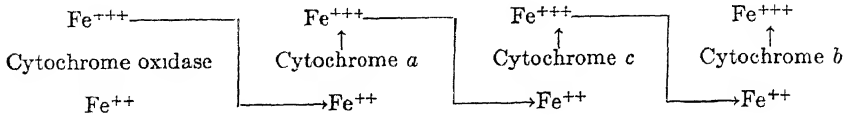
These authors have discovered a new flavoprotein (cytochrome *c* reductase), which reacts with both oxidized cytochrome *c* and reduced triphosphopyridine nucleotide.

† The cytochromes are all iron-containing compounds.

‡ Somewhat resembling oxyhemoglobin (p. 259).

$$Fe^{++}O_2.\text{cytochrome oxidase} \xrightarrow{+H^+} Fe^{+++}\text{cytochrome oxidase} + H_2O$$

The ferric cytochrome oxidase reacts with ferrous cytochrome *a*, oxidizing the latter and being reduced itself. Such reactions proceed with cytochromes *c* and *b*:



The succinate-fumarate system has already been referred to under the metabolism of carbohydrates (p. 327). Summarizing this system, we may state it thus:

$$\text{? Flavoprotein (oxidized)} \xrightarrow{-2H} \underset{\text{Malate}}{\text{Oxalacetate}} \xrightarrow{2H} \underset{\text{Succinate}}{\text{Fumarate}} \xrightarrow{2H} \text{Cytochromes, etc.}$$

Not all biological oxidations require such elaborate systems. The simplest of all types is that where the metabolite, activated by dehydrogenase, reacts directly with molecular oxygen, with no "carrier" intervening:

$$\underset{\text{(Dehydrogenase)}}{\text{Metabolite}} \longrightarrow O_2$$

These reactions are illustrated by the oxidation by tissues (notably the liver) of aldehydes and a number of the purines. Acetaldehyde, for example, is changed to acetic acid. Hypoxanthine is changed to xanthine and finally to uric acid. In these cases, the hydrogen which is removed from the metabolite combines with the oxygen. Sometimes the metabolite needs cytochrome as a "go-between":

$$\text{Metabolite} \longrightarrow \text{Cytochrome} \longrightarrow \text{Oxygen}$$

and sometimes the other "go-between" or "carriers" which have been mentioned.

We shall now discuss some of these various substances.

Dehydrogenases.*—These are enzymes, protein in nature, and rather specific in character. Together with the coenzyme, hydrogen in the metabolite is "activated," removed, and transferred to the coenzyme.

Dehydrogenases, according to Sumner, may be divided into three groups: (a) those which require coenzyme I or coenzyme II (p. 391); (b) those which transfer hydrogen to cytochrome (p. 394); and (c) the yellow enzymes (p. 392).

Under (a) may be cited the conversion of lactic acid to pyruvic acid. A specific dehydrogenase (lactic dehydrogenase), found in heart

* Also called apodehydrogenases.

tissue, etc., is needed for this reaction, and the enzyme cooperates with coenzyme I.

Again, in the conversion of glucose to gluconic acid (p. 18), a specific dehydrogenase (glucose dehydrogenase), found in liver and yeast, cooperates with coenzyme II.

Under (b)—dehydrogenases which transfer hydrogen to cytochrome (p. 394)—may be mentioned succinic dehydrogenase as an example. This enzyme, in the presence of the cytochrome system, converts succinic acid to fumaric acid (p. 327), a reaction, which it may be recalled, is an important step in carbohydrate metabolism (p. 327).

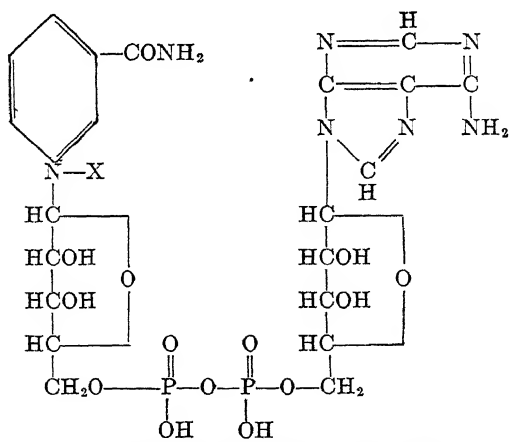
Under (c)—the yellow enzymes—these are proteins containing prosthetic groups (p. 401) related to riboflavin (p. 153). An example of its action is given on p. 393.

Pyridine Nucleotides.—These include coenzyme I and coenzyme II.

Coenzyme I has also been called codehydrogenase I, cozymase, diphosphopyridine nucleotide and Co I. Coenzyme II has also been called codehydrogenase II, triphosphopyridine nucleotide and Co II.

Both coenzymes are nicotinamide adenine dinucleotides; the one difference being that Co I contains two molecules, and Co II, three molecules of phosphoric acid. Nicotinic acid, and its amide, have already been discussed under vitamins (p. 159). The importance of these substances as "building stones" for the respiratory enzymes now becomes clear.

The structural formula for Co I (diphosphopyridine nucleotide) is as follows:



Co I (Diphosphopyridine nucleotide).

which means that it consists of nicotinamide (p. 392), adenine (p. 85), pentose (*d*-ribose, p. 80), and phosphoric acid.

The Co II or triphosphopyridine nucleotide may be represented as follows, though there is some uncertainty as to the exact location of one of the phosphoric acid groups:

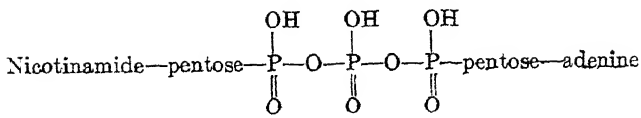
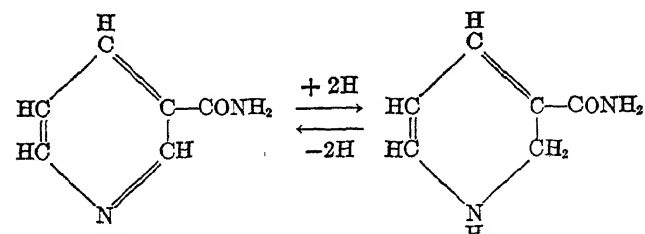


Table 51 gives some of the sources of these two enzymes.

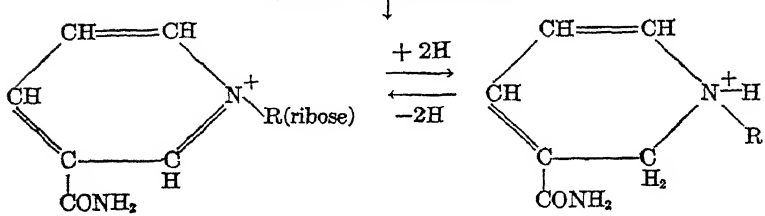
TABLE 51.—SOME EXAMPLES OF THE OCCURRENCE OF Co I AND Co II. [Schlenk, *Symposium on Respiratory Enzymes* (1942)]

Material.	*Coenzyme content in micrograms per gram of fresh material.* Co I	Co II
Yeast	>500	<10
Erythrocytes (horse)	100	>12
Liver (rat)	>200	30
Muscle (rat)	200	50
Kidney (rat)	160	40

These coenzymes, as has already been stated, take up and then give up hydrogen. They are oxidation-reduction systems. This taking up and giving up hydrogen is the property of the nicotinamide part of the molecule and may be represented, in simplified form, as follows:



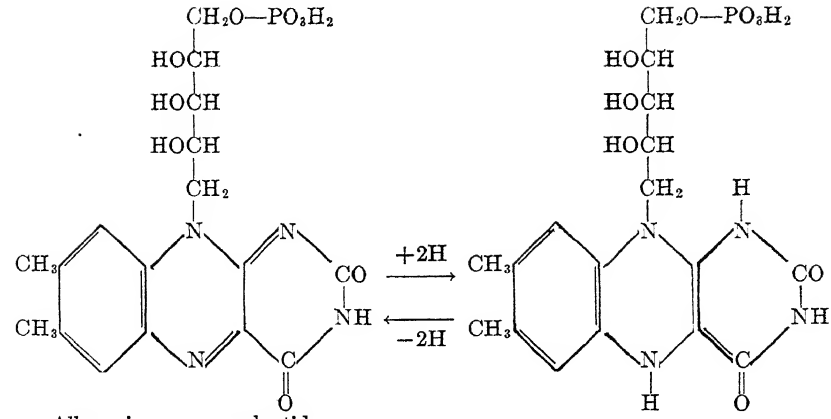
(or, as part of the molecule)



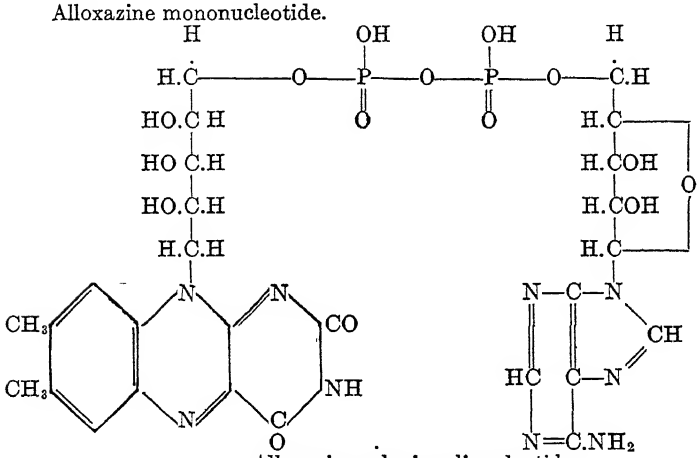
These pyridine nucleotides are directly connected with the vitamin nicotinic acid (or its amide) (p. 159). It has been shown, for example, that a dog or pig suffering from a deficiency of nicotinic acid exhibits a lowered coenzyme I content of the liver and muscle.

Yellow Enzymes (Flavoproteins).—These enzymes are protein compounds, containing, as prosthetic groups, alloxazine mononucleotide (riboflavin phosphate, p. 154) and alloxazine adenine dinucleotide (which is composed of both riboflavin phosphate and adenylic acid).

The structure of these two substances is here given, and the oxidizing-reducing property—the property of taking up and giving up hydrogen—is indicated:



Alloxazine mononucleotide.



Alloxazine adenine dinucleotide.

The alloxazine mononucleotide, or riboflavin phosphate, together with a protein, has also been called Warburg's "yellow enzyme" and was the first of these yellow enzymes isolated. The newer "yellow enzymes" belong to the dinucleotide type.

The close connection between the vitamin riboflavin (p. 153) and these yellow enzymes has been shown by creating a riboflavin deficiency in the rat and noting a decrease in the *d*-amino acid oxidase (p. 353) of the liver and the kidney. The addition of riboflavin to the diet increases the enzyme content of the tissues.

These yellow enzymes are found in animal tissues and in yeast.

The *d*-amino acid oxidase which acts on *d*-amino acids (p. 353) has, as its prosthetic group, alloxazine adenine dinucleotide. This is also true of the xanthine oxidase, which acts on xanthine and other purines (p. 380) and of glycine oxidase, which acts on glycine (p. 360).

In these cases we are dealing with different enzymes probably having the same prosthetic group but joined to different proteins.

An illustration of Warburg's "yellow enzyme" is in the oxidation of hexose-phosphate to phosphohexonic acid—a reaction which is

relatively simple and does not involve the cytochrome system. The system may be formulated as follows:

$$\underset{\text{(Metabolite)}}{AH_2} + \underset{\text{(Coenzyme)}}{F} \rightarrow FH_2 + A$$

[In presence of dehydrogenase]

$$FH_2 + \underset{\text{(Yellow enzyme)}}{Y} \rightarrow F + YH_2$$

$$YH_2 + O_2 \rightarrow Y + H_2O_2$$

The enzymes have been extracted in a high state of purity; and Warburg has shown that these substances *in vitro* can oxidize hexose-phosphate (hexosemonophosphate) into phosphohexonic acid:

$$\underset{\text{(Hexosephosphate)}}{R.CHO} + H_2O + \underset{\text{(Coenzyme)}}{F} \rightarrow \underset{\text{(Phospho-hexonic acid)}}{R.COOH} + FH_2$$

[In presence of dehydrogenase]

FH_2 is not auto-oxidizable (that is, molecular oxygen cannot oxidize it), but it reduces the "yellow enzyme" to a compound which is auto-oxidizable:

$$FH_2 + \underset{\text{(Yellow enzyme)}}{Y} \rightarrow F + YH_2$$

$$YH_2 + O_2 \rightarrow Y + H_2O_2$$

The Szent-Györgyi Cycle.—Szent-Györgyi has advanced the hypothesis that succinic acid and a series of closely related four-carbon compounds (fumaric, malic, and oxalacetic acids) all act as important "carriers." He was led to this view by a number of observations, of which two can be stated here. One observation is that not only do most tissues oxidize succinic acid to fumaric acid, but the former is oxidized more rapidly than any other substance known. The other observation is that malonic acid, which is a specific "poisoner" for succinic acid oxidation, very definitely poisons respiration altogether, even when added in minute quantities.

Cytochrome.—This "carrier" is widely distributed in tissues. It is an iron-pyrrole compound of the heme type, combined with a protein which is not globin. In other words, it is a "hemochromogen" (p. 259).

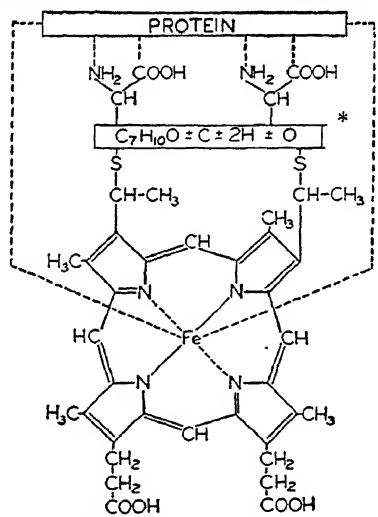
The pigment shows a characteristic absorption spectrum in the reduced form, which fact lends itself to the identification of the substance. Three distinct absorption bands are obtained, identified by the letters *a*, *b*, *c*. These are probably three distinct compounds, though only one of these, cytochrome *c*, has been extensively studied, because of its relative stability. The oxidized modification shows an indefinite spectrum.

By making use of such spectroscopic observations, changes in the cytochrome present in the intact cell may be followed. When, for example, yeast cells (or animal tissues) suspended in water are examined, we can identify the three bands due to cytochrome. If air be passed through the suspension, the bands disappear and reappear again if nitrogen be substituted for the air.

Cytochrome, then, is reduced by the cell, and again oxidized in the presence of oxygen. The reduction of the cytochrome is catalyzed by an enzyme which has been called *cytochrome reductase*, and its oxidation, on the other hand, is catalyzed by the enzyme known as *cytochrome oxidase* (indophenol oxidase), which is, according to Keilin, identical with Warburg's respiratory enzyme. This cytochrome oxidase, more strictly speaking, helps in the reaction between cytochrome *c* and molecular oxygen.

In support of the need for catalytic activity, it may be pointed out that the factors which inhibit the activity of dehydrogenase in the cell inhibit the reduction of cytochrome. Again, cells of yeast, muscle, and other tissues contain an indophenol oxidase which catalyzes the reaction of *p*-phenylenediamine to form the indophenol blue. It can be shown that this same oxidase catalyzes the oxidation of cytochrome. The activity of the oxidase and the oxidation of cytochrome are inhibited by the same substances: for example, potassium cyanide and hydrogen sulfide.

Theorell has suggested the following structure for cytochrome *c*:



Structure of cytochrome *c*.

As an example of cytochrome action, the conversion of succinic to fumaric acid may be cited. The cycle of reactions is probably the following:

1. $\underset{\text{Succinic acid}}{\begin{matrix}CH_2.COOH\\ |\\ CH_2.COOH\end{matrix}} \underset{\text{(Succinic dehydrogenase)}}{\rightleftharpoons} \underset{\text{Fumaric acid}}{\begin{matrix}CH.COOH\\ \|\\ CH.COOH\end{matrix}} + 2H$

2. $2H + 2\ \text{Cytochrome}\ C \rightleftarrows \text{Reduced cytochrome}\ C + 2H^+$

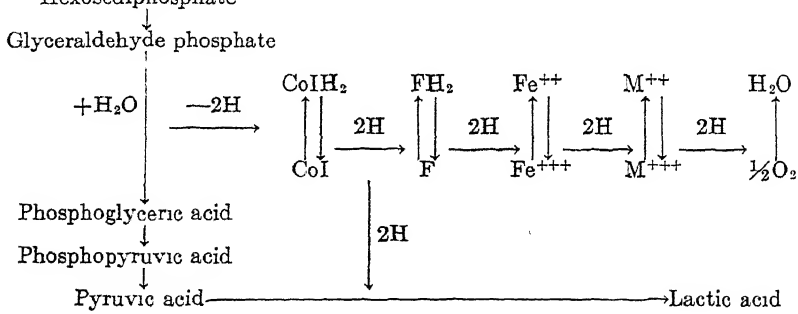
3. $2\ \text{Reduced cytochrome}\ C + 2H^+ + \tfrac{1}{2}O_2 \xrightarrow[\text{2 Cytochrome}]{\text{(Cytochrome oxidase)}} C + H_2O$

* There is uncertainty as to the exact number of C, H and O atoms.

An Application of the Theory of Biological Oxidation.—How does such a theory work in a specific case? How can we, for example, apply it to the metabolism of carbohydrates?

In referring back to p. 324, we see that hexosediphosphate is changed to lactic acid. During the course of these reactions, glyceraldehyde phosphate is oxidized to phosphoglyceric acid. This oxidation, indicated by the letter "O," might also be indicated by "—2H," or the removal of hydrogen (a dehydrogenase reaction). What happens to this "2H"? Here is where our biological system of step-wise oxidations come into play. Table 52 in simplified form, attempts to summarize the facts:

TABLE 52.—(Adapted from Potter, *Medicine*, **19**, 461. The removal of hydrogen requires a dehydrogenase CoI = Coenzyme I. $CoIH_2$ = coenzyme I in reduced form F = flavin compound, FH_2 its reduced form. Fe^{++} and Fe^{+++} = the prosthetic groups of cytochrome. M = group of cytochrome oxidase.)



If we wish to introduce the 4-carbon cycle of Szent-Györgyi—about which there is some question—we might consider the change from triosephosphate to phosphoglyceric acid a change which involves an oxidation. This may be summarized thus:

(1) Triosephosphate + CoI → phosphoglycerate + $CoIH_2$
(2) $CoIH_2$ + oxalacetate → CoI + malate
(3) Malate + CoI → oxalacetate + $CoIH_2$
(4) $CoIH_2$ + fumarate → CoI + succinate

(5) Succinate + oxidized cytochrome *c* → fumarate + reduced cytochrome *c*
(6) Reduced cytochrome *c* + $\frac{1}{2}O_2$ → oxidized cytochrome *c* + H_2O
Reaction 1 + 2 + 3 + 4 + 5 + 6 = 1 + 4 + 5 + 6 =

(7) Triosephosphate + $\frac{1}{2}O_2$ → phosphoglycerate + H_2O

and, Reaction 4 + 5 + 6 =

(8) $CoIH_2 + \frac{1}{2}O_2 \rightarrow CoI + H_2O$
(Potter, *J. Biol Chem.*, **134**, 417.)

"The whole energy of oxidation," writes Szent-Györgyi, "is liberated in the oxidation of hydrogen. . . . We thus have the whole energy cycle: the plant cell, with its chlorophyll, stores the energy of the sun, separating the elements of water, fixing the hydrogen to a solid carbon chain and sending the oxygen back to the atmosphere. All cells cover the energy need by reversing the process, taking the

hydrogen from the organic molecule and uniting it again with the oxygen of the air."

There are a number of oxidases which do not, as yet, fit into a well-defined system of biological oxidations. These will now be discussed.

Catalase.—This enzyme catalyzes the decomposition of hydrogen peroxide into water and molecular oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The enzyme is found in various plant and animal tissues. It is an iron-porphyrin-protein complex (p. 258) and has been obtained in crystalline form (Fig. 95).

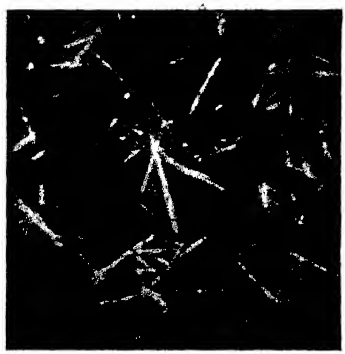


Fig. 95.—Crystals of horse liver catalase; × 300. (Sumner, Dounce and Vernon, *J. Biol. Chem.*, **136**, 353.)

We know little about the physiological importance of catalase. One theory is that it decomposes hydrogen peroxide whenever the latter tends to accumulate in the body. *d*-Amino acid oxidase, xanthine oxidase, glycine oxidase, etc., form H_2O_2 during the course of their activity. For example, in the conversion of glycine to glyoxylic acid by glycine oxidase, the following series of reactions have been formulated:

$$\begin{array}{l} \mathrm{CH_2.COOH} \\ | \\ \mathrm{NH_2} \end{array} + \underset{(E)}{\text{oxidized enzyme}} \xrightarrow{(-2H)} \begin{array}{l} \mathrm{CH.COOH} \\ \| \\ \mathrm{NH} \end{array} + \underset{(EH_2)}{\text{reduced enzyme}}$$

(Imino compound intermediate)

$$\begin{array}{l} \mathrm{CH.COOH} \\ \| \\ \mathrm{NH} \end{array} + H_2O \rightarrow \begin{array}{l} \mathrm{CHO} \\ | \\ \mathrm{COOH} \end{array} + NH_3$$

(Glyoxylic acid.)

$$\underset{(EH_2)}{\text{Reduced enzyme}} + O_2 \rightarrow \underset{(E)}{\text{oxidized enzyme}} + H_2O_2$$

To protect the body against the toxic effects of H_2O_2 the catalase comes into play.

This is an attractive hypothesis, but it still needs much confirmation.

Keilin and Hartree have suggested the following mechanism for catalase activity:

1. $4Fe^{+++} + 2H_2O_2 \rightarrow 4Fe^{++} + 4H^+ + 2O_2$
2. $4Fe^{++} + 4H^+ + O_2 \rightarrow 4Fe^{+++} + 2H_2O$

In reaction (1) the H_2O_2 is reduced by the iron in the catalase (Fe^{+++}) to Fe^{++}, with the liberation of two molecules of water. In reaction (2) the reduced catalase (Fe^{++}) is reoxidized with one molecule of oxygen.

Greenstein, in the course of work on tumor-bearing rats and mice, has made the interesting observation that the catalase activity of the liver of such animals with tumors is decidedly lower than that of normal animals. Furthermore, the removal of the tumor quickly restores the activity to normal.

Peroxidase is also an iron-porphyrin-protein compound (p. 258). This enzyme, found in plant tissues and in milk, spleen, etc., catalyzes the oxidation of various compounds in the presence of hydrogen peroxide. Tryptophan, ascorbic acid, epinephrine and tyrosine, are among compounds of physiological interest which are oxidized by peroxidase. It also oxidizes many diamines (such as *o*-phenylene diamine), phenolic substances (such as guiacum), aromatic monoamines (such as aniline) and indicators (such as phenolphthalein).

Peroxidase can act only in the presence of hydrogen peroxide or certain organic peroxides; but replacing H_2O_2 with molecular oxygen has no effect.

Various iron-porphyrin compounds behave similarly to peroxidase, but the latter is much the more active.

A somewhat modified form of peroxidase, called *verdoperoxidase* by its discoverer, Agner, has been isolated from leucocytes, where it occurs in relatively high concentration. The enzyme is green in color (hence its name) and its absorption spectrum differs from that of the usual peroxidase; but it also reacts with H_2O_2, though not so vigorously as peroxidase. "It is conceivable," writes Agner, "that it has a function in connection with the general reaction of the leucocytes to infection . . . "

The common peroxidase, for which one convenient source is horseradish, has been separated by electrophoresis (p. 265) into two components; and one of these, peroxidase II, was obtained in crystalline form (Theorell).

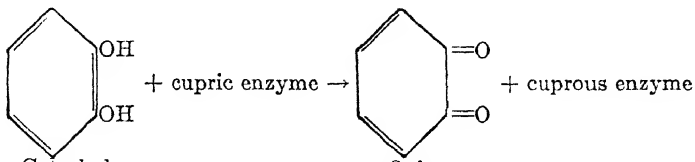
It is worthy of note that such oxidizing enzymes as the cytochromes, catalase and peroxidase are iron-porphyrin compounds joined to protein. This is true of hemoglobin also. Their various physiological activities are probably due to differences in the protein part of the molecule; or perhaps to different combinations with certain groups in the protein molecule. This question is largely one of speculation at present.

Several enzymes are copper-protein complexes of the hemocyanin type (p. 45). They are polyphenol oxidase, laccase and monophenol oxidase (tyrosinase). The relation of these enzymes to hemocyanin, the copper pigment of marine animals, is similar to the relation of the

enzymes of the iron-porphyrin variety (such as cytochromes, cytochrome oxidase, catalase and peroxidase) to hemoglobin.

Polyphenol oxidase (potato oxidase), a variety of which is found in the potato, catalyzes the oxidation of *o*-diphenols (such as catechol or epinephrine) in the presence of molecular oxygen.

The action on catechol occurs in two stages:



Catechol *o*-Quinone

Cuprous enzyme + O_2 → cupric enzyme.

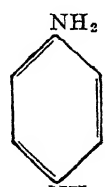
Hydrogen peroxide is not needed in this reaction.*

This enzyme will oxidize monophenols, such as phenol, but very slowly.

Keilin has isolated a polyphenol oxidase from mushrooms; 10 mg. of the purified material was obtained from 15 kg. of the raw material.

How important a constituent the copper is in the make-up of this enzyme is seen by removing the copper from the molecule (by dialysis): the copper-free protein shows no oxidase activity. When copper ions are added the activity is restored. (The copper ions alone also show no activity.) Metals such as iron, cobalt, nickel, manganese or zinc cannot take the place of the copper.

Laccase, another copper-protein enzyme, is found in the lacquer tree and also catalyzes the oxidation of different polyphenols. But it is different from the polyphenol oxidase in the following: laccase is not poisoned by carbon monoxide, whereas polyphenol oxidase (P.O.) is strongly inhibited in its action; laccase readily oxidizes *p*-phenylenediamine, but its oxidation of catechol is slow; the reverse is true of P.O.



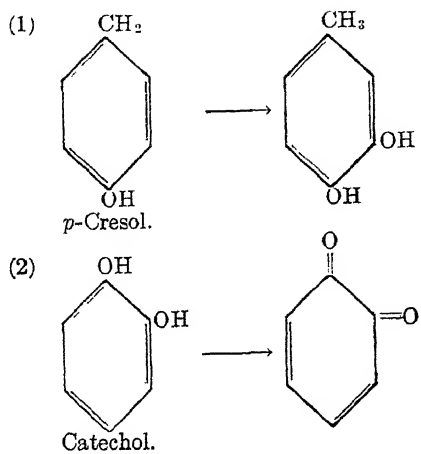
p-Phenylenediamine.

However, there is still some question as to the individuality of laccase.

Monophenol oxidase (tyrosinase), is still another copper-protein enzyme which catalyzes the oxidation of monophenols (such as *p*-cresol, phenol and tyrosine) in the presence of molecular oxygen. The action is not a clear-cut one. Nelson and others have shown that the ordinary,

* The brown (or black) color of the potato when exposed for a time is due to the combination of the quinone with the proteins of the tissue to form melanin-like (?) pigments.

probably impure, tyrosinase catalyzes two types of reaction: (1) one type introduces a hydroxyl group ortho to the one already present in certain monohydric phenols; and (2) the other type brings about the oxidation of certain *o*-dihydric phenols to their corresponding *o*-quinones.



p-Cresol.

Catechol.

This enzyme converts tyrosine into a black pigment (melanin, p. 366).

Ascorbic Acid Oxidase, an enzyme present in plant tissues which catalyzes the oxidation of ascorbic acid, is also a copper-protein compound.

It is assumed that, just as the iron in the iron-porphyrin enzymes undergoes oxidation and reduction during the course of the enzyme's activity, so possibly does the copper in copper-protein enzymes.

It is believed that glutathione and ascorbic acid are both concerned with some biological system of oxidation.

Glutathione is a tripeptide of cysteine, glutamic acid, and glycine (p. 563). It was isolated from tissues by Hopkins in the form of its cuprous salt. The oxidized form, which may be written as G—S—S—G (where G and G stand for oxidized glutathione minus its two sulfur atoms), is readily reduced by tissues to the sulfhydryl form, G.SH; and the latter, in presence of traces of copper, gives up its hydrogen to molecular oxygen, becoming oxidized in turn.

Barron is of the opinion that glutathione maintains enzymes active by keeping their —SH groups (and many possess them) in this (reduced) form.

Ascorbic acid, the antiscorbutic substance, discussed elsewhere (p. 173), is a very active reducing agent and may have a rôle in reactions involving oxidations and reductions in the tissue. It may, in other words, be a "carrier"; but our information, at present, is meager.

Hopkins believes that ascorbic acid may be a coenzyme for the oxidation of glutathione. He finds that the oxidation of ascorbic acid

by oxygen and the ascorbic acid oxidase—obtained from cauliflower—is prevented by the addition of glutathione. He believes that the oxidized ascorbic acid reacts with glutathione, forming ascorbic acid and oxidized glutathione, and that this reaction proceeds faster than the oxidation of ascorbic acid.

Coenzymes and "Carriers."—In the removal of hydrogen from the metabolite, the enzyme known as a *dehydrogenase* must be present. These dehydrogenases are quite specific, and they are named after the substrates on which they act. For example, there is a glucose dehydrogenase, a lactic acid dehydrogenase, and a succinic acid dehydrogenase.

Now, the hydrogen which is removed from the metabolite in the presence of the dehydrogenase combines with a coenzyme, and the latter then passes the hydrogen on to another compound in the chain. In this sense, not only is our coenzyme also a "carrier," but the specific part played by the coenzyme in cooperation with the enzyme (dehydrogenase) becomes clearer.

This view of enzyme-coenzyme coöperation applies at present to biological oxidations only. Phosphate transfer, rather than hydrogen transfer, may also give rise to the equivalent of a coenzyme. In this sense, adenyl pyrophosphate, which is a "carrier" for phosphate groups, alternately gaining and losing such groups (p. 320), may be called a coenzyme.

Prosthetic Groups.—Reference has already been made (p. 95) to a group of enzymes which consist of proteins and groups ("prosthetic" groups) which are not proteins. Roughly speaking, the enzymes which take part in hydrolytic reactions (such as pepsin and trypsin) are presumably just proteins, and the evidence so far is against their having any "prosthetic" group. On the other hand, enzymes active in oxidative reactions are combinations of proteins and prosthetic groups. For summary, we may mention the following enzymes active in oxidations which are known to possess prosthetic groups: cytochrome oxidase, cytochromes, catalase, and peroxidase. In each case, the prosthetic or nonprotein grouping is some variety of heme or iron-porphyrin combination. In the polyphenol oxidase, laccase, tyrosinase and ascorbic acid oxidase, the nonprotein group is a copper-containing compound.

The prosthetic group of the yellow enzymes is riboflavin. Some consider the dehydrogenases to be composed of protein loosely joined to Coenzyme I or Coenzyme II. Some consider cocarboxylase (vitamin B_1 pyrophosphate) to be the prosthetic group of carboxylase (p. 539). If this view is accepted, then we have here an example of a nonoxidative enzyme which contains a prosthetic group.

Nomenclature.—In an effort to bring order out of the confusion resulting from calling one thing by different names—reminding one of the confusion reigning in the field of vitamin B—Dixon classifies the various substances taking part in biological oxidations in the following way:

Warburg School.	*Others.*
Protein	Enzyme, apo-enzyme, dehydrogenase
Enzyme, ferment	Enzyme + coenzyme; holo-enzyme; protein + prosthetic group
Dehydrase	Complete oxidase system, usually containing several enzymes
Partner	Substrate, metabolite
Respiratory enzyme; autooxidizable iron-proteid	Cytochrome oxidase
Yellow enzyme; alloxazine-proteid	Flavoprotein
Pyridine-proteid	Dehydrogenase + Coenzyme I or II
Copper-proteid	Polyphenol oxidase, laccase, tyrosinase, etc.
Protein of aldehyde reductase	Alcohol dehydrogenase
Intermediary enzyme; Zwischenferment	Hexosemonophosphate dehydrogenase
Alloxazine-adenine-proteid	Amino acid oxidase + coenzyme; new "yellow enzyme"
Diphosphopyridinenucleotide	Coenzyme I, cozymase
Triphosphopyridinenucleotide	Coenzyme II

The Transfer of Electrons.—Biological oxidations, involving reactions of oxidation and reduction, should preferably be discussed in terms of a transfer of electrons, in agreement with current chemical practice. For ionization systems involving iron ($Fe^{++} \rightarrow Fe^{+++} + e$) this method is readily adaptable; but its application to organic substances is, at present, not so easy.

Oxidation-Reduction Systems.—Apart from the work so far described, a group of investigators have busied themselves with energy changes involved in biological oxidations, in the ultimate attempt to "predict" whether reactions will "work." In

$$AH_2 + B \rightleftarrows A + BH_2$$

the equation is a reversible one and has a definite equilibrium point under certain given conditions. For the reaction to proceed, B must obviously have a greater tendency to form BH_2 than A to form AH_2. B must be a relatively powerful oxidizing substance in order to succeed. This involves a study of "oxidation-reduction potential."

For example, if we determined the "reduction potentials" of A and B (meaning thereby a quantitative evaluation of their power to give off hydrogen), we ought, under given conditions, to be in a position to predict the course of the reaction. The practical difficulties are, however, great. (See the references at the end of the chapter.)

References

Several volumes on the subject have first to be mentioned. Green is the author of one of these, entitled *Mechanisms of Biological Oxidations* (1940).

In a volume entitled *A Symposium on Respiratory Enzymes* (1942), the following articles should be consulted: Oxidative mechanisms in animal tissues, p. 16 (Ball); hydrogen transport, p. 33 (Potter, Elliott, Ball, Lipmann, Stern, Haas, Stotz); oxidases, peroxidases and catalase, p. 74 (Stern); nicotinamide nucleotide enzymes, p. 104 (Schlenk); flavoproteins, p. 134 (Hogness); cytochromes, p. 149 (Stotz).

Another volume, *Respiratory Enzymes*, edited by Elvehjem and Wilson, with contributions from members of the staff of the Univ. of Wisconsin, covers much the same field. The chapters are the following: Historical Introduction, p. 1 (Elvehjem); dehydrogenases, p. 20 (Potter); oxidases, catalase and peroxidase, p. 38

(Lipton, Arnold, Berger); coenzymes, p. 71 (Baumann, Stare); cytochrome, p. 93 (Burris); flavoproteins, etc., p. 104 (Stark, Gordon, Christensen); inhibition of dehydrogenases, p 137 (Cohen); hydrogen transport systems, p. 158 (Schneider); oxidation-reduction potentials, p. 168 (Axelrod, Johnson), physicochemical theory of enzyme action, p. 203 (Wilson).

Vol. 7 (1939), of the *Symp. Quant. Biol.*, is rich in contributions to biological oxidations. Refer to oxidation-reduction equilibria, p. 1 (Clark); free radicals, p. 33 (Michaelis); flavoproteins, p. 100 (Ball); cytochrome oxidase and cytochrome, p. 111 (Stotz); cytochrome oxidase, p. 121 (Hogness); cytochrome and yellow ferments, p. 130 (Urban); ascorbic acid, p. 137 (King); tyrosinase, p. 148 (Nelson); iron-porphyrin compounds, p. 154 (Barron).

Sumner and Somers, *Chemistry and Methods of Enzymes* (1943), devote several chapters to oxidizing enzymes· chapters 11 (coenzymes I and II); 12 (dehydrogenases), 13 (yellow enzymes); 15 (various oxidases).

Perspectives in Biochemistry (1937), edited by Needham and Green, includes the following articles on oxidation which should be consulted: Respiratory Carriers (Dixon), p. 114; Intermediary Hydrogen-Transport in Biological Oxidations (Krebs), p. 150; Oxidation and Fermentation (Szent-Györgyi), p. 165; Reconstruction of the Chemical Events in the Living Cell (Green), p. 175. That suggestive book by Holmes, *The Metabolism of Living Tissues* (1937), Chaps. 4 and 5, should also be consulted See, also, a chapter on vegetable oxidases by Szent-Gyorgyi, in his *On Oxidation, Fermentation, Vitamins, Health and Disease* (1939).

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Pyridine nucleotides (coenzymes). Axelrod and Elvehjem, *J. Biol. Chem.,* **131,** 77 (1939); Axelrod, Madden and Elvehjem, *Ibid.,* **131,** 85 (1939).

A detailed discussion of coenzymes I and II will be found in the article by Schlenk, *Adv. Enzym.,* **5,** 207 (1945).

Proof that the carbohydrate in coenzyme I is *d*-ribose is supplied by Schlenk, *J. Biol. Chem.,* **146,** 619 (1942).

Flavoproteins. Axelrod, Sober and Elvehjem, *J. Biol. Chem*, **134,** 749 (1940).

For a study of glycine oxidase, a flavoprotein, see Ratner, Nocito and Green, *J. Biol. Chem.,* **152,** 119 (1944).

Cytochromes. Keilin and Hartree, Proc. R. S. (London), Series B, **127,** 167 (1939); Theorell and Åkesson, *J. Am. Chem. Soc.,* **63,** 1804, 1812, 1818, 1820 (1941); Haas, Horecker and Hogness, *J. Biol. Chem.,* **136,** 747 (1940); Haas, Harrer and Hogness, *Ibid.,* **143,** 341 (1942).

Cytochrome oxidase consists of two components, according to Haas, *J. Biol. Chem.,* **152,** 695 (1944).

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Cytochrome reductase is described by Haas, Harrer and Hogness, *J. Biol. Chem.,* **143,** 341 (1942).

Catalase. Sumner, Dounce and Frampton, *J. Biol. Chem.,* **136,** 343 (1940); Dounce, *Ibid.,* **143,** 497 (1942); Keilin and Hartree, Proc. R. S. (London), Series B, **124,** 397 (1938).

For the isolation of catalase from liver, together with a study of some of its chemical properties, see Lemberg, *Biochem. J.,* **37,** 117 (1943).

Greenstein's observations on catalase activity in tumor bearing rats will be found in the *J. National Cancer Institute,* **2,** 525 (1942).

Laccase. Keilin and Mann, *Nature,* **143,** 23 (1939); Proc. R. S. (London), Series B, **125,** 187 (1938).

Ascorbic acid oxidase. Lovett-Janison and Nelson, *J Am Chem. Soc.*, **62,** 1409 (1940); Powers, Lewis and Dawson, *J. Gen. Physiol.*, **27,** 167 (1944).

Glutathione Barron and Singer, *Science,* **97,** 356 (1943).

Peroxidase. Keilin and Mann, Proc. R S (London), Series B, **122,** 119 (1937) The oxidation products obtained by the action of peroxidase on *p*-cresol have been studied by Westerfeld and Lowe, *J. Biol. Chem*, **145,** 463 (1942).
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CHAPTER 20

ENERGY METABOLISM

WHEN foodstuffs (metabolite) and oxygen unite, heat is evolved. The metabolite contains carbon, hydrogen, oxygen, often nitrogen, sometimes phosphorus, sulfur, etc. Most of these elements are completely oxidized in the body. The carbon and hydrogen, for example, are oxidized to carbon dioxide and water; the sulfur and phosphorus, to sulfate and phosphate, respectively. The nitrogen is not completely oxidized. In the human being, this element is eliminated very largely as urea; and since this substance contains carbon and hydrogen, it cannot be said that all the carbon and all the hydrogen are burned to carbon dioxide and water. Likewise, not all of the sulfur is eliminated as sulfate; some may appear as "neutral sulfur" (p. 469).

In any event, what should be stressed is that, except for nitrogen and sulfur, the elements which have been discussed can be oxidized as completely in the body as outside of it; and the result of such an oxidation is the "production" of heat—a very necessary result in so far as the activities of living matter are concerned.

It is no small tribute to the genius of Lavoisier that he connected his theory of oxidation with the general process of respiration in man.

The Temperature of the Body.—The temperature of a normal person (measured by mouth) is in the neighborhood of 37° C. This temperature varies imperceptibly, irrespective of weather. Not only is heat produced, but there must also be some heat-regulating mechanism whereby fluctuations in temperature are prevented.

Mammals and birds, the "warm-blooded" animals, show such constancy of temperature, irrespective of outside conditions. The reptiles, amphibia and fishes, the "cold-blooded" animals, show temperature fluctuations dependent upon environmental conditions. Their heat-regulating mechanism is either less efficient, or, for more profound reasons, it may not be needed. Hibernating animals also show a temperature in accord with their surroundings; and during this period, their heat-regulating mechanism seems to function little.

The fact that the temperature of the body is 37° C. (98.6° F) and that it remains so indefinitely (for all practical purposes), is a clear indication that heat is being produced. But the *quantity* of such heat production can be measured not by a thermometer, but by a calorimeter.

Calorimeter.—Calorimeters are of two kinds: the one measures the fuel value of coal or food, and the other measures the heat evolved by the body. We shall deal first with the former type.

The principle of the calorimeter—the "bomb" calorimeter of Berthelot—is easily understood. The fuel (or food) is in a steel cylinder filled with oxygen. The reaction is started by a platinum wire

heated by an electric current. The heat evolved in the reaction is communicated to a weighed quantity of water surrounding the cylinder. Without going into details, we can say that knowing the weight of the water and the increase in temperature, the heat evolved by the fuel (or food), measured in calories, can be readily calculated

Heat is measured in calories. The *calorie* used in physics represents that amount of heat necessary to increase the temperature of 1 gm. of water 1° C. (from 15°–16° C). The calorie used in nutritional studies is the large *Calorie* (spelled with a capital *C*)—also written as kg. cal. and Kcal.—representing the amount of heat necessary to increase the temperature of 1 kg. of water 1° C. The Calorie, then, is equivalent to 1000 small calories. We shall use the Calorie (C) exclusively.

Returning to our calorimeter, if the quantity of water is represented by 1 kg., and the increase in temperature is 1° C., the heat produced by the burning of the foodstuff would be 1 C(alorie). Similarly,

4 kg. of water raised 1° C. corresponds to 4 C
1 kg. of water raised 4° C. corresponds to 4 C
2 kg. of water raised 2° C corresponds to 4 C

The calorific value, then, is obtained by multiplying the weight of the water (in kilograms, in our case) by the increase in temperature (in °C).

In this way we arrive at the following average values:*

1 gm. of carbohydrate when burned yields 4 1C.
1 gm. of fat when burned yields 9 4 C.
1 gm of protein when burned yields 5 6 C.

These are the values obtained when such foodstuffs are burned in the calorimeter. Of the three foodstuffs, the carbohydrates and fats are burned as completely in the body as in the calorimeter; but this is not true of the protein. Here its nitrogen is not eliminated as such, but in the form of urea and several other nitrogenous products. Making due allowance for such incomplete combustion in the body, the fuel value of 1 gm. of protein, in so far as the body is concerned, is nearer 4.1 C. Or, in general, to use round figures,

1 gm. of carbohydrate yields 4 C.
1 gm. of fat yields 9 C.
1 gm. of protein yields 4 C.

The Animal Calorimeter.—This apparatus, developed by Atwater, Rosa, and Benedict, is adapted for measuring the heat evolved by an individual (*direct* calorimetry), and for measuring the oxygen intake and carbon dioxide and nitrogen output, from which the amount of protein, carbohydrate, and fat metabolized can be calculated; and these figures also supply data for calculating the heat produced (*indirect* calorimetry).

Several details of the calorimeter—known as a respiration calorimeter (see Fig. 96)—include a lighted and furnished room in which the individual (the subject) may remain in comparative comfort, with walls of metal and wood to prevent heat loss. In the room are pipes

* These figures are very approximate.

containing water—with the flow carefully regulated—which take up the heat given off by the body. The amount of heat can be calculated by knowing the temperature of the water as it enters the room, the temperature when it leaves it, and the rate of flow of the water

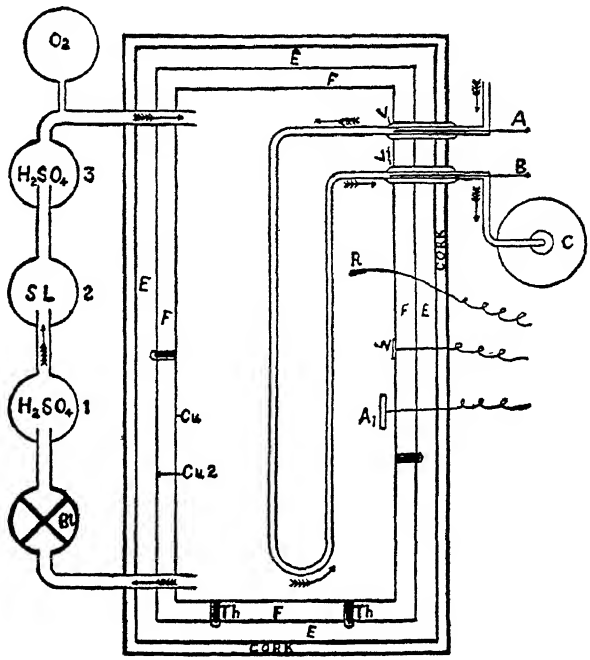


Fig. 96.—Schematic diagram of the Atwater-Rosa-Benedict respiration calorimeter. (Lusk, *Science of Nutrition*, W. B. Saunders Co.)

Ventilating System:
- O_2, Oxygen introduced as consumed by subject.
- 3, H_2SO_4 to catch moisture given off by soda lime.
- 2, Soda lime to remove CO_2.
- 1, H_2SO_4 to remove moisture given off by patient.
- Bl, Blower to keep air in circulation.

Indirect Calorimetry:
- Increase in weight of H_2SO_4 (1) = water elimination of subject.
- Increase in weight of soda lime (2) + increase in weight of H_2SO_4 (3) = CO_2 elimination.
- Decrease in weight of oxygen tank = oxygen consumption of subject.

Heat-absorbing System:
- A, Thermometer to record temperature of ingoing water.
- B, Thermometer to record temperature of outgoing water.
- V, Vacuum jacket.
- C, Tank for weighing water which has passed through calorimeter each hour.
- W, Thermometer for measuring temperature of wall.
- A_1, Thermometer for measuring temperature of the air.
- R, Rectal thermometer for measuring temperature of subject.

Direct Calorimetry:
- Average difference of A and B × liters of water + (gm. water eliminated × 0.586) ± (change in temperature of wall × hydrothermal equivalent of box) ± (change of temperature of body × hydrothermal equivalent of body) = total calories produced.
- Th, thermocouple; Cu, inner copper wall; Cu_2, outer copper wall; E, F, dead air spaces.

Using a closed circuit (see Fig. 97), a measured amount of air is drawn in by a pump, and the eliminated carbon dioxide and water are

absorbed and weighed. Measured quantities of oxygen are added when needed. Provision is also made for the collection of urine and feces, with the main object of determining the amount of nitrogen eliminated.

Oxygen.—How and under what conditions the oxygen of the air reaches the cells of the body via the blood have already been discussed (pages 298–301). Some abnormalities may be stressed at this point.

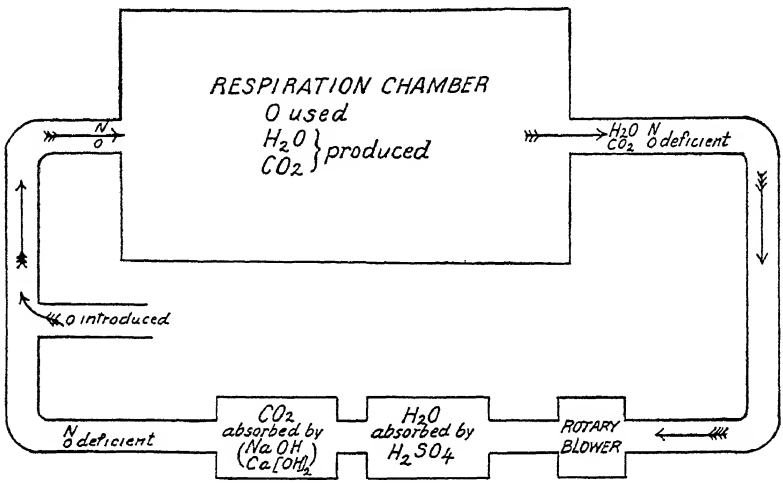


Fig. 97.—Diagram of circulation of air through respiration apparatus. (Atwater and Benedict)

An anoxia (oxygen deficiency) may develop at times. For example, at high altitudes "mountain sickness" may result, due to diminished oxygen tension in the air, and hence in the alveolar air and blood stream.

A common clinical form of anoxia is observed in diseases of the respiratory tract, as in pneumonia, for example. Here there is an interference with oxygen absorption.

Another common decrease is seen in cases of anemia, due to a diminished amount of hemoglobin, and hence not so much oxygen can combine with the blood pigment as under normal conditions.

Poisoning due to carbon monoxide interferes with respiration, due to the preferential combining capacity of hemoglobin with carbon monoxide rather than with oxygen.

Certain drugs will cause a methemoglobinemia. Such substances oxidize hemoglobin to methemoglobin, which cannot combine with oxygen. The extent of active respiration will depend upon the amount of hemoglobin which has not been changed.

These cases can be multiplied.

The Respiratory Quotient.—For reasons which will become apparent as we proceed, the value of the "respiratory quotient" (R. Q.) must be known. By the R. Q. is meant the volume of carbon dioxide evolved divided by the volume of oxygen consumed.

When carbohydrates are oxidized, the reaction may be represented (using glucose as a type):

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

$$\text{Here the R.Q.} = \frac{6CO_2}{6O_2} = 1$$

Fats, containing less oxygen in their molecules than carbohydrates, would need more oxygen from the outside for complete combustion:

$$C_{57}H_{104}O_6 + 80O_2 \rightarrow 57CO_2 + 52H_2O$$

Triolein.

$$\text{R.Q.} = \frac{57CO_2}{80O_2} = 0.71$$

Since the formula of a protein is unknown, and therefore an equation such as the above cannot be written, the R. Q. must be determined in an indirect way.

According to Loewy, quoted by Lusk, the analysis of 100 gm. of meat protein gives the following figures (in grams):

	C	H	O	N	S
	52.38	7.27	22.68	16.65	1.02
of which is eliminated in the urine:	9.406	2.663	14.099	16.28	1.02
and in the feces:	1.47	0.212	0.889	0.37	
leaving for the respiratory process:	41.50	4.40	7.69		
deducting intramolecular water:		0.961	7.69		
leaving behind	41.50	3.439			

To oxidize 41.5 gm. of carbon and 3.439 gm. of hydrogen we require 138.18 gm. of oxygen and 152.17 gm. of carbon dioxide are produced.

The *weights* must now be converted into *volumes* in order to get the R. Q. of proteins. One gm. of oxygen, at standard conditions, occupies a volume of 0.699 liter; and 1 gm. of carbon dioxide occupies a volume of 0.5087 liter.

For oxygen, then, $138.18 \times 0.699 = 96.63$ liters, and for carbon dioxide, $152.17 \times 0.5087 = 77.39$ liters.

$$\text{The R. Q. of protein} = \frac{77.39}{96.63} = 0.801.$$

When 1 gm. of nitrogen (in the form of urea, etc.) is eliminated, it means the following: 1 gm. of urinary nitrogen corresponds to 6.25 gm. of protein, and represents the absorption of 5.92 liters of oxygen, the elimination of 4.75 liters of carbon dioxide, and the production of 26.51 Calories (Lusk).

In severe diabetes, the R. Q., which normally on a mixed diet may be around 0.85, may reach a figure as low as 0.7, indicating that much of the combustion is derived from fats. One of the many striking results of insulin treatment is that the R. Q. increases, showing that carbohydrates (and part of the protein molecule) are being better utilized for energy purposes.

Indirect Calorimetry.—By knowing the oxygen consumption and the carbon dioxide output, and the amount of nitrogen (as urea, etc.)

eliminated, it is possible to calculate the amount of carbohydrate, fat, and protein consumed, and the amount of heat (in Calories) produced Here the heat is not measured directly and the apparatus employed is relatively simple.

Bodansky (*Physiological Chemistry* [1938], p. 517) gives the following instructive example: During a twenty-four-hour period, the subject consumed 400 liters of oxygen, and eliminated 340 liters of carbon dioxide and 12 gm. of nitrogen (as urea, etc.).

The amount of protein represented by 1 gm. of nitrogen (1 × 6.25 gm. protein) requires for oxidation 5 92 liters of oxygen, and 4.75 liters of carbon dioxide are eliminated.

TABLE 53.—THE SIGNIFICANCE OF THE NONPROTEIN RESPIRATORY QUOTIENT AS REGARDS THE HEAT VALUE OF 1 LITER OF OXYGEN, AND THE RELATIVE QUANTITY IN CALORIES OF CARBOHYDRATE AND FAT CONSUMED. (Zuntz and Schumberg, Modified by Lusk, Modified by McClendon.)

One Liter of Oxygen is Equivalent to

Nonprotein respiratory quotient.	Grams.		Calories.
	Carbohydrate.	Fat.	
0.707	0.000	0.502	4.686
0 71	0.016	0.497	4.690
0.72	0.055	0.482	4.702
0.73	0 094	0.465	4.714
0.74	0.134	0.450	4.727
0.75	0.173	0 433	4.739
0.76	0 213	0.417	4.751
0.77	0.254	0 400	4.764
0.78	0.294	0.384	4.776
0.79	0.334	0.368	4.788
0.80	0.375	0 350	4.801
0.81	0 415	0.334	4.813
0 82	0.456	0.317	4.825
0 83	0.498	0.301	4.938
0 84	0.539	0 284	4 850
0.85	0.580	0 267	4.862
0 86	0.622	0 249	4.875
0.87	0.666	0 232	4.887
0 88	0 708	0 215	4.899
0 89	0.741	0 197	4.911
0 90	0.793	0.180	4.924
0.91	0.836	0.162	4.936
0 92	0 878	0 145	4.948
0.93	0.922	0.127	4.961
0 94	0.966	0.109	4.973
0 95	1.010	0.091	4.985
0 96	1.053	0.073	4.998
0 97	1.098	0.055	5.010
0.98	1.142	0.036	5.022
0.99	1.185	0.018	5.035
1.00	1.232	0.000	5.047

Since 12 gm. of nitrogen were eliminated, this would mean 12 × 5.92 = 71 liters of oxygen (approximately), and 12 × 4.75 = 57 liters of carbon dioxide, as being due to protein.

The oxygen consumption due to carbohydrate and fat is 400 −

71 = 329 liters; and the production of carbon dioxide due to carbohydrate and fat is 340 − 57 = 283 liters.

$$\text{The R. Q. of } \underset{\text{(nonprotein)}}{\text{carbohydrate + fat}} = \frac{283}{329} = 0.86.$$

From Table 53 we gather that when the R. Q. of "nonprotein" is 0 86, 1 liter of oxygen is equivalent to 0.622 gm. of carbohydrate and 0.249 gm. of fat.

Therefore, 329 liters of oxygen mean

$$329 \times 0.622 = 204 \text{ gm of carbohydrate}$$
$$\text{and } 329 \times 0.249 = 82 \text{ gm of fat}$$
$$\text{and 12 gm. of nitrogen eliminated mean } 12 \times 6\ 25 = 75 \text{ gm. of protein}$$

In other words, during this twenty-four-hour period, the subject utilized 204 gm. of carbohydrate, 82 gm. of fat, and 75 gm. of protein

The heat value of these foodstuffs can now be calculated very approximately:

$$\begin{aligned} 75 \times 4 &= 300 \text{ Calories from protein} \\ 82 \times 9 &= 738 \quad \text{“} \quad \text{“ fat} \\ 204 \times 4 &= 816 \quad \text{“} \quad \text{“ carbohydrate} \\ &\overline{1854} \text{ Calories} \end{aligned}$$

Basal Metabolism.—The total energy output is the resultant of two factors. Under normal conditions, one of these factors is a fairly constant one. It represents the energy needed in maintaining the temperature of the body (in warm-blooded animals), in maintaining the heart beat, etc. The other factor is a widely fluctuating one, depending upon the extent of exercise and upon the amount of food consumed. A subject who has fasted for some twelve hours before the experiment and who is in a state of complete rest during the experiment, will give off an amount of heat which will tend to be constant. The heat output, under these conditions, is called the "basal metabolism," and it is measured in terms of the number of Calories produced per square meter of body surface per hour.

In one form of basal metabolic test, the amount of oxygen consumed is measured. Under the conditions of the experiment, for each liter of oxygen consumed, 4.8 C. of heat is generated.

If, for example, the individual consumes 1 l. oxygen in four minutes, or 15 l. in one hour, the heat generated would be 15 × 4.8, or 72 C. per hour.

If, depending upon height and weight (Fig. 98), the individual has a surface area of 2 square meters, each square meter will give off 36 C. of heat.

The number "36" represents Calories per square meter of surface per hour.

The importance of surface area was first emphasized by Rubner. The variations obtained by recording metabolic experiments per unit of weight are quite large.

DuBois has developed an empirical formula relating surface area, height and weight, and the nomogram makes the estimation of surface area very simple (Fig. 98).

The average basal metabolic rate for normal adults (between thirty and forty years) is 39.5 C. for males and 36.5 C for females.*

To calculate the basal metabolic rate based on heat production, we shall take an example from Cantarow and Trumper.†

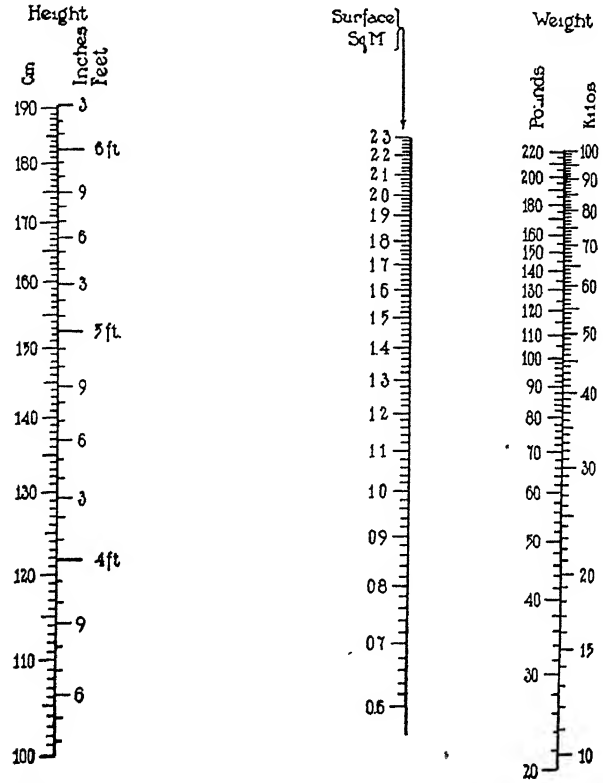


Fig. 98.—Nomogram permitting direct estimation of surface area from height and weight by DuBois' formula $A = H^{0.725} \times W^{0.425} \times 71.84$ A = surface area in square centimeters, H = height in centimeters, and W = weight in kg (Sq cm. = sq. m $\times \frac{1}{10000}$) The surface area is found at the point of intersection of the middle scale with a straight line drawn from the observed height on the left hand scale to the observed weight on the right hand scale. (Peters and Van Slyke, *Quantitative Clinical Chemistry*, Williams and Wilkins Co.)

The individual is a male, aged thirty-five, height 67 inches, weight 154 pounds. His surface area is 1.8 square meters.

The actual oxygen consumption is 200 ml. per minute, or 12 l. per hour.

For every 1 l. of oxygen consumed, 4.825 C. of heat is generated.

Therefore, the heat expenditure is $12 \times 4.825 = 57.9$ C. per hour.

Since the patient's surface area is 1.8 square meters, the Calories per square meter per hour $= \frac{57.9}{1.8} = 32.16$ C.

* 124 "normal "college women gave an average basal metabolism of 33.4 C (McCreery, Lamb and Bavousett).

† See reference at the end of the chapter.

The normal value, according to DuBois, is 39.5 C. per square meter per hour.

Therefore, the basal metabolic rate =

$$\frac{39\,5 - 32\,16}{39.5} \times 100 = -18.5 \text{ per cent;}$$

which is 18.5 per cent below normal.

Variations in Basal Metabolism.—Variations due to age are given in Table 54. The most striking variations are observed in thyroid disease. "It must be admitted," write DuBois and Chambers, "that the basal metabolism tests are seldom of great value except in the diagnosis and treatment of the thyroid gland . . . "

In severe cases of hyperthyroidism, increases of 75 per cent (and more) above normal have been obtained; and in hypothyroidism (myxedema), figures representing 40 per cent below normal are not uncommon.

TABLE 54.—STANDARD VALUES FOR CALORIES PER SQUARE METER PER HOUR. (Boothby, Berkson and Dunn, *Am. J. Physiol.*, **116**, 468.)

Males.		Females.	
Age last birthday.	Mean.	Age last birthday.	Mean.
Years		*Years*	
6	53.00	6	50.62
7	52.45	6½	50.23
8	51.78	7	49.12
8½	51.20	7½	47.84
9	50.54	8	47.00
9½	49.42	8½	46.50
10	48.50	9–10	45.90
10½	47.71	11	45.26
11	47.18	11½	44.80
12	46.75	12	44.28
13–15	46.35	12½	43.58
16	45.72	13	42.90
16½	45.30	13½	42.10
17	44.80	14	41.45
17½	44 03	14½	40.74
18	43.25	15	40.10
18½	42.70	15½	39.40
19	42.32	16	38.85
19½	42.00	16½	38.30
20–21	41.43	17	37.82
22–23	40.82	17½	37.40
24–27	40.24	18–19	36.74
28–29	39.81	20–24	36.18
30–34	39.34	25–44	35.70
35–39	38.68	45–49	34.94
40–44	38.00	40–54	33.96
45–49	37.37	55–59	33.18
50–54	36.73	60–64	32.61
55–59	36.10	65–69	32.30
60–64	35.48		
65–69	34.80*		

* Obtained by extrapolation.

In fevers (pneumonia, typhoid, etc.) the metabolic rate is increased by about 13 per cent for each 1° C.

In prolonged starvation, covering a period of some ten days or more, the metabolic rate is definitely reduced (Table 55).

TABLE 55.—BASAL METABOLISM IN PROLONGED FASTING IN MAN. (Lusk, *Science of Nutrition.*)

	Day of fast and of postfast.	Weight, kg.	Calories in 24 hours	
			Total.	Per sq. m.
Prefast average		63.1	1517	904
Fast	3	63.0	1545	920
	10	59.1	1282	786
	19	56.1	1177	729
	29	53.4	1095	702
	40	50.0	978	639
Postfast	4	50.4	1110	726
	7	52.0	1306	830
	11	53.9	1285	819
	18	58.4	1466	899
	33	64.6	1570	923

TABLE 56.—THE INFLUENCE OF DIET AND MECHANICAL WORK UPON THE METABOLISM OF A MAN 61 TO 63 KG. IN WEIGHT

Diet and conditions.	Heat produced.			Heat lost.		
	Twenty-four hours.	Increase.	Increase due to work.	Evap. H_2O	Rad. and cond.	Work.
	Calories	*Per cent*	*Calories*	*Calories*	*Calories*	*Calories*
No food, rest	1976	. . .	. . .	380	1596	
Cane sugar 600 gm. + H_2O 3000 gm., rest	2023	+ 2.4	. . .	529	1494	
Same + work	2868	+45.2	845	907	1727	234
Protein, large amount of meat, rest	2515	+27.2	. . . .	614	1901	
Protein, same diet, + work	3370	+70.5	855	1235	1901	234

Of course, muscular work increases considerably the energy requirements above the basal metabolic level. In Table 56, above, prepared by Rubner, a comparison is made between the effect on heat production of (*a*) the addition of carbohydrate and protein; (*b*) work. None of the heat production due to work is derived from the specific dynamic action of the foodstuffs (see below).

The effect of certain drugs on the basal metabolic rate has been much investigated. This applies more particularly to phenol derivatives of the type of 2,4-dinitrophenol and 4,6-dinitro-*o*-cresol. The injection of 3 mg. per kilogram weight of the former substance in-

creases the basal metabolic rate tenfold. In this respect, its effect is comparable to thyroxine. However, in myxedema, such phenols will restore the basal rate without influencing the other symptoms of the disease; which is in striking contrast to the effect of thyroxine.

Specific Dynamic Action.—When protein equivalent to 100 C. is ingested, heat equivalent to 130 C. is produced. This action of protein in stimulating metabolism Rubner called the "specific dynamic action" (S. D. A.) of protein. Fats and carbohydrates given in equivalent quantities (that is, in amounts to produce 100 C.) produce 113 C. and 105 C., respectively. These are Rubner's figures; but they are considered too high. In any case, the most striking effects are obtained with protein; and here the results are the same whether the protein is taken in as food, or whether its constituent amino acids are injected. It is true that the kidney does work in excreting urea and ammonia derived from the protein (or amino acid) ingested; but Borsook asserts that this accounts for less than one-half the observed increase in metabolism. Nor can the result be due to increased gastro-intestinal activity; for the feeding of bones and meat extract, which gives rise to much intestinal irritation, has no effect on S. D. A.

TABLE 57.—ENERGY METABOLISM, AND URINARY NITROGEN, SULFUR, AND URIC ACID FOLLOWING THE INGESTION OF 87 GM. GELATIN. (Borsook, *Biological Reviews*, 11, 147.)

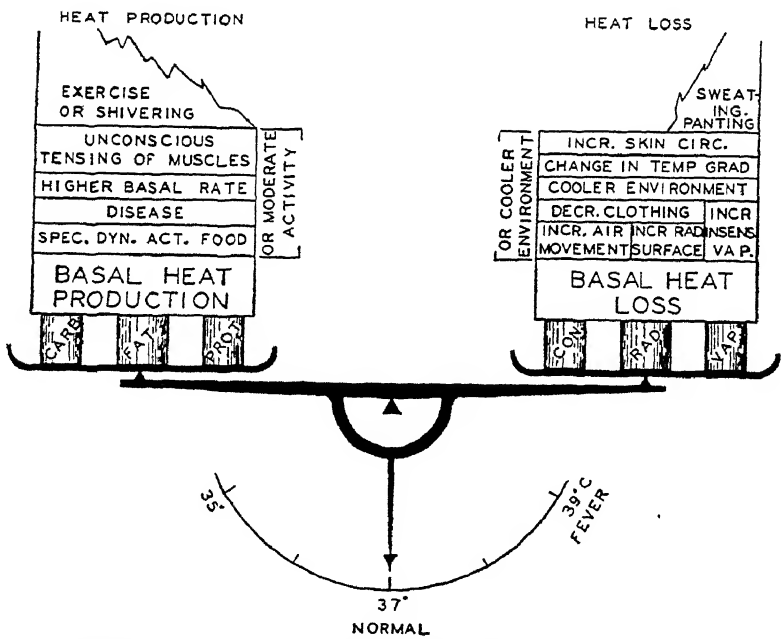
Time.	Calories kg. cal. per hour.	Urinary nitrogen, gm.	Total urinary sulfur, mg.	Total urinary uric acid, mg.
6.30 A. M.	62.9			
6.00–7.10	..	..	..	17.1
8.00	..	..	..	13.5
8.30	77.2			
9.00	..	0.60	29.6	35.8
9.30	80.4			
10.00	..	0.83	31.8	42.7
10.30	80.5			
11.00	..	0.98	36.9	40.9
11.30	75.5			
12.00 NOON	..	1.14	41.6	36.5
12.30 P. M.	73 4			
1.00	..	0.87	38.2	20.9
1.30	70.0			
2.00	..	0.78	45.6	20.8
2.30	70.7			
3.00	..	0.76	51.1	23.1
3.30	69.8			
4.00	..	0.73	49.0	21.1
4.30	70.0			
5.00	..	0.65	49.9	13.3
5.30	60.1			
6.00	..	..	..	19.2

The individual amino acids all show powerful S. D. A. effects, although they vary among themselves.

In an experiment dealing with the effect of the ingestion of gelatin, Borsook showed that there was not only an increase in energy metabolism, but also an increase in the excretion of urinary nitrogen, sulfur, and uric acid (Table 57).

That the liver may be concerned with the process is made probable by Wilhelmj's observation that, after hepatectomy, glycine and alanine show no S. D. A.

Borsook expresses S. D. A. as the ratio of the Calories in excess of the basal to urinary nitrogen in excess of the basal. The variation in the S. D. A. of protein, he explains, can be interpreted on the basis of a new theory of specific dynamic action: the S. D. A. is a composite of two factors, the one nearly constant and represented by the increased energy production resulting from the metabolism and excretion of nitrogen (7–10 C. per gram of nitrogen); and the other, a more variable and, usually, larger fraction, arising from the metabolism of carbon.



ig. 99.—Balance between factors increasing heat production and heat loss. (DuBois, *Harvey Lectures*, Ser. 34, p. 88, Williams and Wilkins Co.)

Apparently, the amino acids do not stimulate cellular metabolism. The increase in metabolism is presumably the result of the deamination of these amino acids.*

Heat Regulation.—Under normal conditions, there is, as it were, a balance of forces between the extent of heat produced and the extent of heat lost (Fig. 99). In order that the temperature may remain constant, the equilibrium will shift either to the right or to the left.

* For a criticism of these views, see Forbes, etc. (references at the end of the chapter).

The manner in which heat is lost from the body, as well as its extent, is summarized by Vierordt (quoted by Howell):

Through urine and feces	1.3	per cent or	48 C.
By expired air; warming of air	3.5	" " "	84 C.
Vaporization of water from lungs	7.2	" " "	182 C.
Evaporation from skin	14.5	" " "	364 C.
Radiation and conduction from skin	73.0	" " "	1792 C.
		Total daily loss =	2470 C.

While such figures may change with changes in environmental conditions, in general it may be said that loss of heat is due, in the main, to evaporation and radiation. To some extent, such heat loss is controlled by clothing. But the important regulatory mechanism is an automatic reflex control (through sweat nerves and vasomotor nerves).

The heat production, on the other hand, is dependent upon the extent of cellular oxidation; and this, in turn, is partly dependent upon the amount of muscular activity and upon the food eaten (S. D. A. of foodstuffs). But there is also an involuntary control, in the shape of an involuntary reflex on muscular metabolism. For example, as the outside temperature is lowered, the heat production is increased, although the temperature of the body does not change. This is termed "chemical regulation" by Rubner.

Energy Requirements.—Roughly speaking, the energy requirement is the resultant of two factors: a fairly constant factor, as represented by the basal metabolism; and a variable factor, depending upon physical activity. The effect due to S. D. A. must also be taken into account.

An exhaustive study of energy needs has been made by Stiebling and Ward. In preparing "adequate diets," they have constructed a "standard" table, giving not only the calorific needs at various ages and varying activity, but also the amounts needed of protein, calcium, phosphorus and iron (Table 58).

TABLE 58.—DIETARY "STANDARDS" PER CAPITA PER DAY. (Stiebling and Ward *U. S. Dept. Agricul.*, Circular 296.)

	Energy.	Protein.	Calcium.	Phosphorus.	Iron.
	Cal.	*gm.*	*gm.*	*gm.*	*gm.*
Child under 4 years	1200	45	1.00	1.00	.006–.009
Boy, 4–6; girl, 4–7 years	1500	55	1.00	1.00	.008–.011
Boy, 7–8; girl, 8–10 years	2100	65	1.00	1.00	.011–.015
Boy, 9–10; girl, 11–13 years	2400	75	1.00	1.20	.012–.015
Moderately active woman; boy, 11–12; girl, over 13 years	2500	75	1.00	1.20	.013–.015
Very active woman; active boy, 13–15 years	3000	75	0.88	1.32	0.015
Active boy over 15 years	3000–4000	75	0.88	1.32	0.015
Moderately active man	3000	67	0.68	1.32	0 015
Very active man	4500	67	0 68	1 32	0 015

Four dietary plans were drawn up: (*a*) restricted diet for emergency use, allowing for a narrow margin of safety; (*b*) adequate diet

at minimum cost; (*c*) adequate diet at moderate cost; and (*d*) liberal diet.

The needs during one week on diet (*a*) of a family of 5, consisting of 2 moderately active adults, and 3 children, two, five, and thirteen years old, respectively, are: 14 quarts of milk, 9 eggs, 2½ pounds of

TABLE 59

Foods or nutrients.	Restricted diet for emergency use.	Adequate diet at minimum cost.	Adequate diet at moderate cost.	Liberal diet.
Per year				
Grain products,* lbs.	240	224	160	100
Dried beans, peas, nuts, lbs.	30	30	20	7
Potatoes and sweet potatoes, lbs.	165	165	165	155
Milk, quarts (or equivalent)	155	260	305	305
Lean meat, poultry, fish, lbs.	30	60	100	165
Eggs, dozen	8	15	15	30
Tomatoes and citrous fruits, lbs.	50	50	90	110
Leafy green and yellow vegetables, lbs.	40	80	100	135
Dried fruits, lbs.	10	20	25	20
Other vegetables and fruits, lbs.	40	85	210	325
Fats (oils, butter, bacon, salt pork), lbs.	45	49	52	52
Sugars, lbs.	50	35	60	60
Per day				
Calories	2675	2980	2985	2930
Fat, gm.	87	115	130	149
Carbohydrates, gm.	398	397	370	310
Calcium, gm.	0.85	1.28	1.26	1.27
Phosphorus, gm.	1.34	1.72	1.58	1.61
Iron, gm.	0.0111	0.0134	0.0144	0 0152
Vitamin A units	2746	5067	5692	6495
Vitamin C units.	86	118	168	206
Calories from protein, percentage.	11	12	11	12
Protein from animal products, percentage	25	39	47	66

* The annual per capita grain consumption between 1925 and 1929 was 220 pounds gross (including a 20 per cent waste) or 187 pounds net. In this table only 5 per cent waste is allowed.

TABLE 60.—PERCENTAGE DISTRIBUTION OF FOODS ON THE FOUR DIETS.

Foodstuffs.	Restricted diet for emergency use.		Adequate diet at minimum cost.		Adequate diet at moderate cost.		Liberal diet.	
	Monetary distribution	Calorie distribution.	Monetary distribution.	Calorie distribution.	Monetary distribution.	Calorie distribution.	Monetary distribution.	Calorie distribution.
Grain products, dried beans, and potatoes	20	43	15	35	10	24	5–7	15
Dairy products	25–30	12	30–35	18	25–30	19	30	19
Vegetables and fruits	20–25	14	20–25	15	25–30	18	30	18
Lean meat, fish, and eggs	10	5	15	8	15–20	12	25–30	21
Fats, sugars, accessories	20	26	15	24	15–20	27	5–7	27

meat, 4 pounds of butter and other fats, 2¼ pounds of dried beans and peas, 3 quarts of tomatoes, 3 pounds of cabbage and similar green or yellow vegetables, 16 pounds of potatoes, 24 pounds of flour and cereals, and 4½ pounds of sugar and other sweets.

TABLE 61 —ENERGY EXPENDITURE PER HOUR UNDER DIFFERENT CONDITIONS OF MUSCULAR ACTIVITY (Sherman, *Chemistry of Food and Nutrition.* By permission of The Macmillan Company, Publishers.)

Form of activity.	Calories per hour.		
	Per 70 kg. (average man).	Per kg.	Per pound.
Sleeping	65	0.93	0.43
Awake lying still	77	1.10	0 50
Sitting at rest	100	1.43	0.65
Reading aloud	105	1.50	0.69
Standing relaxed	105	1.50	0.69
Hand sewing	111	1.59	0.72
Standing at attention	115	1.63	0.74
Knitting (23 stitches per minute on sweater)	116	1.66	0.75
Dressing and undressing	118	1.69	0.77
Singing	122	1.74	0.79
Tailoring	135	1.93	0.88
Typewriting rapidly	140	2.00	0.91
Ironing (with 5-pound iron)	144	2.06	0.93
Dishwashing (plates, bowls, cups, and saucers)	144	2.06	0.93
Sweeping bare floor (38 strokes per minute)	169	2.41	1.09
Bookbinding	170	2.43	1.10
"Light exercise"	170	2.43	1.10
Shoe making	180	2.57	1.17
Walking slowly (2.6 miles per hour)	200	2.86	1.30
Carpentry, metal working, industrial painting	240	3.43	1.56
"Active exercise"	290	4.14	1.88
Walking moderately fast (3.75 miles per hour)	300	4.28	1.95
Walking down stairs	364	5.20	2.36
Stoneworking	400	5.71	2.60
"Severe exercise"	450	6 43	2.92
Sawing wood	480	6.86	3.12
Swimming	500	7.14	3.25
Running (5.3 miles per hour)	570	8.14	3.70
"Very severe exercise"	600	8.57	3.90
Walking very fast (5.3 miles per hour)	650	9.28	4.22
Walking up stairs	1100	15.8	7.18

Using this table, we get

8 hours of sleep at 65 C. per hour	=	520 C.
2 hours light work at 170 C.	=	340 C.
8 hours carpenter work at 240 C.	=	1920 C.
6 hours sitting at rest at 100 C.	=	600 C.
Total daily requirement		3380 C.

The variations in the four types of diet are brought out in Tables 59 and 60.

For a moderately active man, with a daily calorific requirement of 3000, the authors suggest 67 gm. of protein (compare with Table 13, p. 111) The British Ministry of Health, using the same calorific requirement, is more liberal with its protein. It suggests 80 to 100 gm. per day, "of which not less than one third must be of animal origin."

Du Bois and Chambers state that some 1,200 of the 2000–3000 Calories needed per day should come from "protective foods" to supply the minimum necessary quantities of vitamins, calories and essential amino acids. These 1,200 Calories are derived from 1 pint of milk, 1 egg, 1 serving (3–4 ounces) of meat, 3 teaspoons (15 gm) of butter, 4 servings of whole grain bread or cereal, 2 vegetables, other than potato, one of which is raw, and 2 fruits, one of which is raw.

The energy expenditures (per hour) under different conditions of muscular activity are given by Rose in Table 61.

References

One of the early accounts of the "human" calorimeter of Atwater and Rosa is to be found in Bulletin 63, U. S Dept. of Agriculture (1899). A later one, by Atwater and Benedict, is described in a publication of the Carnegie Institution, Washington (1905). See, also, Lusk, *The Science of Nutrition* (1928), Chap. 3.

For a general discussion, with emphasis on clinical values, see the article on "Calories in Medical Practice" by DuBois and Chambers in the *Handbook of Nutrition* (1943), p. 55.

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For a study of the basal metabolism of normal college women, see McCreery, Lamb and Bavonsett, *J. Nutrition*, **25,** 245 (1943).

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The important study by Stiebling and Ward dealing with energy requirements is published as U. S. Dept. of Agriculture, Circular 296 (1933). See, also, Brody in the *Ann. Rev. Biochem.*, **4,** 383 (1935).

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For progress in the field of energy metabolism, see the *Ann. Rev. Physiol.*, **5,** 105 (1943) (Forbes and Voris); **6,** 123 (1944) (Kleiber); **7,** 181 (1945) (Himwich).

CHAPTER 21

INORGANIC METABOLISM. WATER

THE general biological importance of calcium, phosphorus, iron, sodium, potassium, sulfur, and chlorine has been stressed from time to time in these pages. Their presence in the body has been known for some time, and some of their functions have been discovered. More recent work—in many cases involving spectroscopic examination—has revealed the presence of not less than 55 elements in plant or animal tissue. Many of them are present in "traces" only; and the temptation is strong to dismiss such elements by calling them "impurities." While such a conception may apply to some, it probably does not apply to a number of them. In any case, caution is necessary. We shall see presently how effective "traces" of copper are in the utilization of iron.

Sherman gives a list of a number of the more important elements (with their percentages), found in the human body (Table 62).

TABLE 62.—APPROXIMATE ELEMENTARY COMPOSITION OF THE BODY. (Sherman and Lanford, *Essentials of Nutrition*, Macmillan)

Element.	*Percentage.*
Oxygen	65.
Carbon	18
Hydrogen	10
Nitrogen	3.
Calcium	2.[a]
Phosphorus	1.1[b]
Potassium	0.35
Sulfur	0.25
Sodium	0.15
Chlorine	0.15
Magnesium	0.05
Iron	0.004
Manganese	0 00013
Copper	0.00015
Iodine	0.00004
Cobalt	*c*
Zinc	*c*
Others of more doubtful status	

[a] Estimates vary widely.
[b] Percentage varies with that of calcium.
[c] Believed to be essential, but quantitative data are not yet at hand.

As showing how many more elements in "traces" there may be, the results of a spectrum analysis of milk ash by Drea may be cited. He has been able to detect the following elements in addition to "common" ones: aluminum, barium, boron, chromium, fluorine, lead, lithium, molybdenum, silver, rubidium, silicon, strontium, tin, titanium, vanadium, and zinc. In other tissues—in plant as well as animal tissues—we find, in addition, cobalt, nickel, selenium, bromine, bismuth, arsenic, etc.

The functions of some of the mineral elements which have been

studied are fairly clear. A number, such as calcium and phosphorus, are constituents of bone and teeth; some, such as iron, sulfur, and phosphorus, are necessary elements in important organic compounds found in the body; still others, such as sodium chloride, serve as electrolytes; and it is possible that some, like copper, play a rôle as catalysts.

Not only are such elements in themselves important but equally important is their relationship to one another. The relationship of calcium and phosphorus in rickets has already been referred to (Chap 8) and will be stressed once again. But brief mention may be made here of several other elements. When a frog's heart is immersed in solutions containing several salts at various concentrations, it has been found that the heart will beat normally provided the ratio $\frac{K^+}{Ca^{++}}$ is what is found in blood. In dealing with the effect of ionic concentrations upon the irritability of tissues, Holmes points out that the irritability depends very largely upon the ratio.

$$\frac{Na^+ + K^+ + OH^-}{Ca^{++} + Mg^{++} + H^+}$$

and that when the concentration of the ions in the numerator is increased, there is an increase of irritability; whereas the reverse is true if the ionic concentration in the denominator is increased. "Ringer's solution," so often used to retain the activities of tissues and tissue slices, is made up of a solution of chlorides of potassium, sodium, calcium, and magnesium in concentrations comparable to those in blood.

We shall now take up some of the elements individually. In these discussions, the effects of the elements are really their effects as *constituents of salts.*

Calcium (See Chap. 22).—As the chief constituent of bone, calcium is present as a salt resembling minerals of the apatite group; 99 per cent of the total amount of calcium in the body is found in the bones. The element probably exists as a double salt of the carbonate and phosphate, $CaCO_3.nCa_3(PO_4)_2$, where n is not less than 2, nor greater than 3. There is also evidence that the principal inorganic salt in bone may be a hydroxyapatite.

In the blood, calcium is found almost exclusively in the plasma, where it occurs to the extent of about 9 to 11.5 mg. per 100 cc. of serum. The relationship [Ca] × [P] = 36 (where [Ca] and [P] are expressed in milligrams per 100 cc.) holds fairly well. Some 60 per cent of the calcium in the blood is in a diffusible form, and the remainder is quite nondiffusible. The nondiffusible portion is probably attached to the serum albumin.

It is probable that almost all of the diffusible calcium in the blood is in the ionic form, and the actual amount seems to be dependent upon the amount of protein.

A concentration of ionic calcium below the normal amount, brought about by a deficiency of the hormone in the parathyroid, affects the central nervous system and produces an increased irritability of the peripheral nerves. At a later stage, muscle spasms (including the face, hands, and feet) and general convulsions make their appearance. We have here an example of tetany which can be cured by an extract of parathyroid glands (p. 483).

A somewhat different picture of the results of calcium deficiency is obtained when the diet is very low in calcium—10 mg. per 100 gm. of food—and when the animal has been kept on such a diet for a sufficiently long time (nine weeks). Such a diet—dealing with the rat—leads to muscular weakness and collapse rather than to tetany.

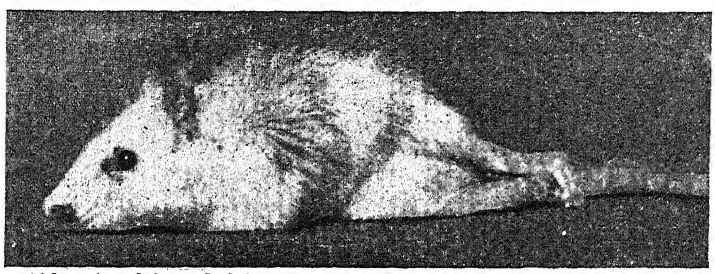

Fig. 100.—A calcium-deficient rat photographed when it had been nine weeks on the deprivation regime, six days after the induction of paralysis by galvanic stimulation. Note the helpless paralytic condition of the hind limbs, the encrusted nares, the size and complete prostration of the rat. This animal died two days later, weighing 82 gm. Hemorrhagic areas were observed in the lungs, brain, liver, intestines, and gluteal muscle. (Boelter and Greenberg, *J. Nutrition*, **21**, 61.)

The animal suffers from a low calcium rickets (osteoporosis, an abnormal porousness of bone) and is very susceptible to hemorrhages. Such hemorrhages and their consequences of prostration and paralysis may be induced by a mild galvanic shock (Fig. 100).

In this deficiency there is a decrease in the serum calcium and a decrease in the calcium and magnesium content of the whole carcass.

By supplying food containing an adequate amount of calcium, the animal becomes normal again.

While the common form of rickets is one in which there is a deficiency of phosphorus, calcium may also be involved (Chap. 8). Vitamin D, possibly by regulating the utilization of calcium from the intestine, influences the extent to which the body uses the element.

It appears that the absorption of calcium is increased by increasing the acidity of the intestinal contents; and it also seems that more calcium is absorbed from concentrated than from dilute solutions.

Apart from the importance of calcium in the structure of bone and teeth and in its influence upon the excitability of the motor system, it also plays a rôle in blood clotting (Chap. 13).

Unlike sodium and potassium, much of the calcium is excreted by the bowel. As a rule, some 65 to 75 per cent of the element is found in the feces, and 25 to 35 per cent in the urine.

Sherman is of the opinion that not less than 0.70 gm. of calcium

is the daily need of the adult. From 0.8 to 1.0 gm. represents an optimal rather than a minimal allowance. Milk and cheese are particularly rich sources. Milk contains about 1.4 gm. of calcium per liter; and cheese, 5 to 10 gm. per kg.

The mere fact that a food is rich in calcium—or in any other element—does not necessarily mean that eating such a food will cause 100 per cent absorption and assimilation. Various studies have shown that from 20 to 30 per cent of the calcium in milk is utilized by the human organism. In the case of green vegetables, poor utilization of the element is perhaps due to the presence of oxalic acid which interferes with the absorption of calcium.

Present-day consumption of large quantities of refined cereals, with much of the original calcium in the whole grain lost, and sugar, which is devoid of minerals, makes the problem of supplying mineral needs difficult.

Calcium (and this applies to magnesium too) is poorly absorbed when brown bread is eaten. This is due to the presence in the bread of a complex acid, phytic acid, which combines with the calcium (from the food in general) to form an insoluble product which is not absorbed. The bad effect of this phytic acid may be overcome by adding calcium carbonate to the flour.

Phosphorus (See also Chaps. 7 and 22).—The phosphorus is not only present in inorganic combinations (in bones, teeth, blood, etc.), but in many organic combinations. Among the latter may be mentioned phosphatids, nucleic acid, phosphoprotein (as casein), adenylic acid, coenzyme, yellow enzymes, thiamin phosphate, phosphocreatine, hexosephosphates, and triosephosphates. All of these substances have already been discussed in various sections of the book.

It has been estimated that there is some 700 gm. of phosphorus in the body, of which 600 gm. is found in the skeleton, 57 gm. in muscle, 5 gm. in brain, 2 gm. in blood, etc. The daily needs have been calculated by Sherman to be some 1.32 gm. The foods particularly rich in this element are cheese, nuts, eggs, meat, and milk.

The functions of phosphorus in the body involve bone and tooth formation, the maintenance of acid-base and calcium equilibria, and the metabolism of carbohydrate.

On a diet containing as little as 0.017 per cent of phosphorus, young rats grow slowly for five to six weeks and then decline and die two weeks later. Metabolic studies reveal a large loss of calcium, excreted mainly through the kidneys. Most of the excreted phosphorus is found in the feces.

Using radioactive phosphorus as an "indicator," it has been found that within twenty-seven days, 45 per cent of the phosphorus given is excreted through the kidneys and 11.5 per cent through the bowel.

Magnesium.—This element is an essential constituent of the chlorophyll molecule, and presumably therefore of importance to plant life. That it is also important to animal life has been shown, among others, by McCollum, who found that a diet containing 0.18 mg. of magnesium per 100 gm. of food (but otherwise quite adequate) gives rise, in rats,

to vasodilatation and hyperirritability of the nervous system, resembling, in some ways, the tetany due to calcium deficiency. Under these conditions, while, of course, the amount of magnesium in the blood is subnormal, the amount of calcium remains at its normal level. Nor, in a reverse situation, with a low blood calcium, can tetany be prevented by the addition of magnesium. What is called "tetany" due to magnesium deficiency has characteristics which make it different from "tetany" due to calcium deficiency.

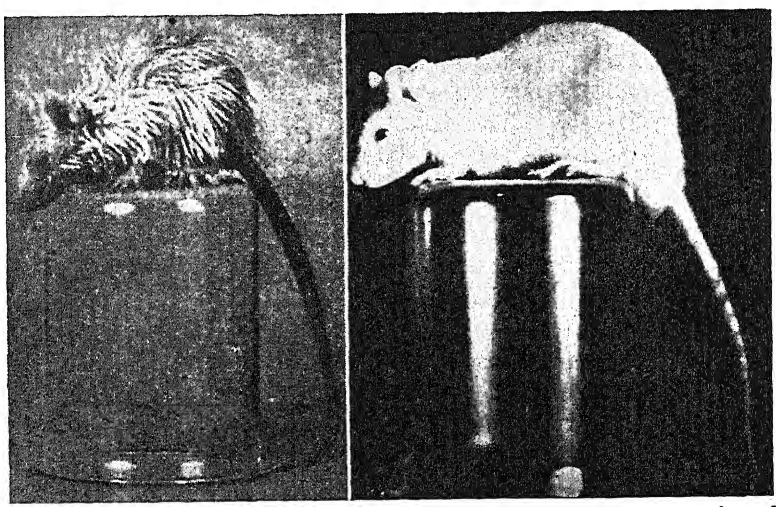

Fig. 101.—*A*, Photograph of a rat after fifty-four days on a magnesium-deficient diet (1.2 mg. of Mg per 100 gm. of food). *B*, The same rat at ninety-one days, after being transferred to the control diet on the sixty-first day. (Tuft and Greenberg, *J. Biol. Chem.*, **122**, 723.)

Greenberg has shown that even on diets containing a higher percentage of magnesium than that of McCollum, rats exhibit symptoms of malnutrition (Fig. 101).

Chicks, apparently, require even more magnesium in their diet than do rats. If the minimum magnesium requirement of the normal rat is about 50 p. p. m. (parts per million), that of the chick is nearer 400 p. p. m. during the first weeks of life.

About 71 per cent of the total magnesium found in the body is located in the bones (in the form of phosphate and carbonate). In the blood we find 1 to 3 mg. per 100 cc. This amount is fairly evenly distributed between serum and cells, in contrast to calcium, which is found almost exclusively in serum.

While the total quantity of magnesium in the body is far less than that of calcium, more of the former is found in muscle than of the latter. On the average, we find about 21 mg. of magnesium per 100 gm. of muscle tissue, as compared to 7 mg. of calcium. The magnesium, according to Lohmann, acts in conjunction with the adenylic acid as a "coenzyme" in carbohydrate metabolism (and also in yeast fermentation).

TABLE 63.—APPROXIMATE AMOUNTS OF CALCIUM, PHOSPHORUS, AND MAGNESIUM IN 100 GM OF EDIBLE FOOD (Schmidt and Greenberg, *Physiol. Rev*, **15**, 300)

	Calcium.	Phosphorus.	Magnesium.
	Gm.	*Gm.*	*Gm.*
Beef (lean)	0.007	0 218	0.024
Eggs	0 067	0.180	0 011
Egg yolk	0.137	0.524	0.016
Milk	0.210	0 093	0 012
Cheese	0.931	0 680	0.037
Wheat	0 045	0 423	0.133
Potatoes	0.014	0.058	0.028
Corn meal	0.018	0 190	0.084
Oranges	0.045	0.021	0.012
Almonds	0.239	0.465	0.251
Spinach	0.067	0 068	0.037
Beans (dried)	0.160	0.470	0.156
Linseed meal	0.413	0.741	0.432
Cotton seed meal	0.265	1.193	0.462

In general, the metabolism of magnesium is not unlike the metabolism of calcium, which, in turn, must be related to that of phosphorus. From one-fifth to one-half of the magnesium is excreted via the urine; the rest appears in the feces.

Like the corresponding salts of calcium and strontium, the magnesium salts of mineral acids produce an acidosis. This is due to the

TABLE 64.—DISTRIBUTION OF ELEMENTS IN THE BLOOD OF HUMAN SUBJECTS IN MILLIGRAMS PER 100 CC. (Schmidt and Greenberg.)

Substance.	*Normal (mean value).*
Total serum calcium	10.3
Diffusible calcium	5 4
Nondiffusible calcium	4 9
Inorganic serum phosphorus	4 0
Whole blood magnesium	4 6
Red corpuscle magnesium	6 6
Plasma magnesium	2 7
Serum magnesium	2 5
Diffusible serum magnesium	1 9

fact that whereas much of the Mg^{++} is excreted with the feces, the acid ion is absorbed and finally excreted in the urine. Magnesium salts are diuretics and cathartics.

Tables 63 to 65 deal with analyses of calcium, phosphorus, and magnesium.

Sodium.—Our main source of this element is the salt (sodium chloride) employed in cooking and seasoning, although some, of course, is derived from the foods we eat. The ordinary daily diet varies from 10 to 50 gm. of sodium chloride.

Sodium (and this is also true of potassium) is very easily absorbed, some 90 to 95 per cent appearing in the urine. It has been claimed by Bunge, and fairly generally accepted, that the addition of an excess

TABLE 65.—COMPOSITION OF BLOOD IN CERTAIN PATHOLOGICAL CONDITIONS. (Schmidt and Greenberg.)

Disease	Substance	Mg per 100 cc.
Rickets (human subjects)	Serum calcium Inorganic phosphate Serum magnesium	9.0 3 0 2.2
Parathyroid tetany (human subjects)	Serum calcium Inorganic phosphate Serum magnesium	7.9 5 0 2 0
Hyperparathyroidism (human subjects)	Serum calcium Inorganic phosphate	15.9 2.2

of sodium to the diet causes an excessive excretion of potassium, and vice versa. The explanation offered as to why herbivorous animals so often have a craving for salt is that their food is particularly rich in potassium, thereby giving rise to an excessive excretion of sodium. Foods of vegetable origin are richer in potassium than in sodium.

Some 93 per cent of the total base in blood serum is due to sodium (Table 66). Together with other salts, it maintains the osmotic pressure and base equilibrium of the blood.

TABLE 66.—CONCENTRATION OF BASES IN BLOOD AND MUSCLE. (Peters and Van Slyke, *Quantitative Clinical Chemistry*. Williams and Wilkins Co, Publishers.)

Substance.	*Blood serum (mg. per 100 cc)*	*Blood cells (mg. per 100 cc.)*	*Muscle (mg. per 100 gm).*
Sodium	335	19	79 9
Potassium	19.5	420	320.1
Calcium	9.6	0 5	7.5
Magnesium	3.2	3	21.2

The extent of the excretion of sodium is dependent upon the amount of intake, and although an animal can maintain itself on surprisingly small quantities—in the rat, as little as 0.1 per cent of sodium chloride in the diet—a minimum amount seems absolutely necessary. Below minimal quantities, there are loss of appetite, retarded growth, disturbance of the reproductive function and ultimate death.

A connecting link between the adrenal cortical hormone and the metabolism of sodium has been established. The removal of the cortical hormone is followed by a considerable loss of the element from the body (p. 503).

The absorption of sodium is so rapid that its radioactive form can be detected in human milk in twenty minutes after ingestion. There is some reason to believe that sodium—and possibly other inorganic elements—are appreciably absorbed through the stomach wall.

Potassium (See also under Sodium, p. 426).—While the ease of absorption and general metabolism resemble those of sodium, each element has very specific functions, since these elements cannot replace one another. It has already been indicated that potassium is found very largely in the cells of the body, whereas sodium is widely distributed in the body fluids (see Table 66). When, however, the cells

are stored at low temperatures, they lose some potassium and take up sodium.

Using radioactive potassium, it can be shown that the element penetrates rapidly into most of the tissues of the body and only a small quantity is found in the plasma.

The growth of rats is definitely retarded when the daily diet contains less than 15 mg. of potassium. These animals develop lesions of the heart and renal hypertrophy

It is claimed that the element is important in carbohydrate metabolism because it catalyzes the transfer of phosphates from 2-phosphopyruvic acid to adenylic acid.

Chlorine (See also under Sodium, p. 426).—In the form of sodium chloride, the chlorine plays a rôle in osmotic pressure relationships and in maintaining the water content of the body. The loss of salt by the body means loss of water.

The chloride concentration of normal human plasma is equivalent to 5.60 to 6.30 gm. (of Cl as NaCl) per liter. A solution of 9 gm. of sodium chloride per liter is isotonic with serum; which means that the chlorides are responsible for two-thirds of the osmotic pressure of the blood.

The "chloride shift" (p. 305) is of importance in acid-base equilibria. It will be remembered, in this connection, that the chloride ion readily passes through the cell membrane, but that the sodium and potassium ions do not.

In tracing the origin of the hydrochloric acid of the stomach, we must ascribe it, to some extent at least, to the sodium chloride of the blood. Changes in gastric acidity involve changes in the composition of the blood and are not influenced by variations in chloride intake.

The metabolism of chlorine cannot be separated from the metabolism of sodium. As has been pointed out, some 10 to 15 gm. of sodium chloride is needed daily. The chlorine (as chloride) is as readily absorbed and metabolized as is the sodium (as sodium chloride); and on a diet deficient in salt, the excretion of chlorine is reduced correspondingly. In one case (cited by Sherman), the excretion dropped from 4.60 gm. per day to 0.17 gm. during the course of thirteen days.

In a suggestive experiment, rats on a synthetic diet with normal chloride content (0.28 per cent) were compared with rats on the same diet, but the chlorides of the salt mixture were replaced by equivalent quantities of the corresponding bicarbonates. The chloride-deficient ration contained 0.02 per cent chloride and 0.49 per cent bicarbonate. At this level of intake, the bicarbonate was considered to be innocuous and the results obtained were attributed to the deficiency of dietary chloride.

In comparison with the rats receiving the normal chloride ration, the chloride-deficient rats showed depression of appetite, increased consumption of water, increased heat production and diminished body gain of nitrogen and energy.

So far as is known, there are no "organic" chlorides in the body. The *p*H of the tissues is such that no combinations of protein-chloride

are possible; so that we have inorganic chlorides, in the form of potassium chloride (in the cell) and sodium chloride (in the plasma).

Iodine.—This element is an essential constituent of the body. It is estimated that of a total of 25 mg. found in the human organism, 15 mg. is in the thyroid. In this organ, a large percentage (but not all) of the iodine is in combination in the organic molecule called thyroxine, a hormone which will be discussed subsequently (Chap. 24).

The concentration of iodine in the thyroid gland is remarkable. Whereas in whole blood the iodine concentration is of the order of one part in 25 million, in the thyroid, it is one part in 2,500.

Using radioactive iodine, it can be shown that the iodine from the blood stream is rapidly transported to the thyroid, where it quickly becomes incorporated in organic molecules—as 3,5-diiodotyrosine and thyroxine (p. 479).

The blood may show values for iodine ranging from 3 to 20 micrograms per 100 cc (1 microgram = 0.001 mg.); and about one-fourth of this amount represents thyroxine.

Since thyroxine influences the rate of oxidations in the body, among other things, and since iodine is a necessary constituent of the molecule, the importance of the element may now be understood.

A deficiency of iodine in the food (and water) may lead to simple (endemic) goiter. In certain inland regions, such as the Great Lakes district in this country, or the Alpine regions in Europe, the water (and the food grown on the soil) may contain less iodine than is necessary for normal well-being. When that happens, goiter in its various stages makes its appearance. The simplest treatment—and a very effective one—is to incorporate a small quantity of iodine, in the form of sodium iodide, in the common table salt. Usually, one part of sodium iodide in 100,000 parts of sodium chloride is sufficient.

It has been estimated that the average person needs 0.05 mg. per day. Since the ocean is relatively rich in iodine, seafoods (fish and oysters) are a good source of this element. Table 67 gives values for iodine in foods.

Bromine.—That bromine is present in animal tissues is beyond question; but just what its function is, if it has any, is a mystery. The claims made, at one time or another, that the metabolism of bromine bears some relation to mental disease, or that there is a bromine-containing hormone in the pituitary, are highly questionable. Winnek and Smith find that a synthetic diet in which the bromine content is less than 5 parts per 10 million supports growth in the rat.

It has been estimated that the blood contains from 0.23 to 1.71 mg. per 100 cc. The content of bromine (in milligrams per 100 gm. of dry weight) in a number of foods is: bread, 0.61 to 0.9, potatoes, 0.27 to 1.42; lentils, 1.02; melons, 9.45 to 26.2. In ordinary salt, for every gram of chlorine there is, it is estimated, 1 mg. of bromine.

Fluorine.—This element is present in various tissues of the body, particularly in bone and teeth. It has been estimated that the normal bone contains from 0.01 to 0.03 per cent of fluorine; and dental enamel, 0.01 to 0.02 per cent.

Since no diet has so far been devised which is free from fluorine, it is difficult to investigate, at present, the function of the element. Mottled enamel, a defect in teeth, endemic in this country and elsewhere, has been attributed to the fluorine contained in the drinking water. The amount must be in excess of 2 parts per million. Such teeth show chalky white patches, and the enamel is frequently pitted and corroded (Fig. 102) Histological examination reveals imperfect

TABLE 67.—IODINE IN FOODS FROM GOITROUS AND NONGOITROUS REGIONS: PARTS PER BILLION OF DRY MATTER. (Sherman, *Chemistry of Food and Nutrition.* By permission of The Macmillan Company, Publishers.)

Kind of food.	Goitrous regions.	Nongoitrous regions.	Authority.
Wheat.	1–6	4–9	McClendon
Oats	10	23–175	"
Carrots	2	170	"
"		507	Okla. Agr. Expt. Sta.
Lettuce		618	" " " "
Potatoes.	85	226	McClendon
"		10–216	Frear
"		90–700	Georgia Agr. Expt. Sta.
Cabbage.		776	Adolph and Whang
Cranberries		26–35	Morse
Asparagus.		946	Okla. Agr. Expt. Sta.
Radishes.		994	" " " "
Tomatoes		379	" " " "
Milk.	265–322	572	Remington and Supplee
Butter.	140	....	McClendon
Sea foods:			
Codfish....		5,350	United States Bureau of Fisheries
Conch		1,140	" " " " "
Crabmeat		1,460	" " " " "
Flounder.		1,480	" " " " "
Oysters.		1,800–3,500	" " " " "
Red snapper		1,440	" " " " "
Salmon		570–2,200	" " " " "
Shrimp.		1,100	" " " " "
Cod liver oil.		7,670	" " " " "
" " "		3,000–13,000	Holmes and Remington

calcification. The minimum quantity of fluorine in water to give rise to mottled enamel is believed to be from 0.72 to 2 mg. per liter. Fluorine (as fluoride), given in relatively large doses, is quite toxic. Eight to 9 mg. of fluorine per kilo of body weight given to cattle produces loss of appetite, disturbed osseous metabolism and fatty degeneration. Studies in oxygen-uptake suggest interference with cellular metabolism.

Some startling facts have appeared linking dental caries with fluorine. It would seem that a certain amount of fluorine in the water—somewhere in the neighborhood of 1.8 parts per million, and therefore less than what would give rise to mottled enamel—protects against dental caries. Where the drinking water contains but a trace of the element, caries is more prevalent. In confirmation of this view, an

analysis of the enamel of sound teeth shows a fluorine value of 0.0111 per cent and that of carious teeth, 0.0069.

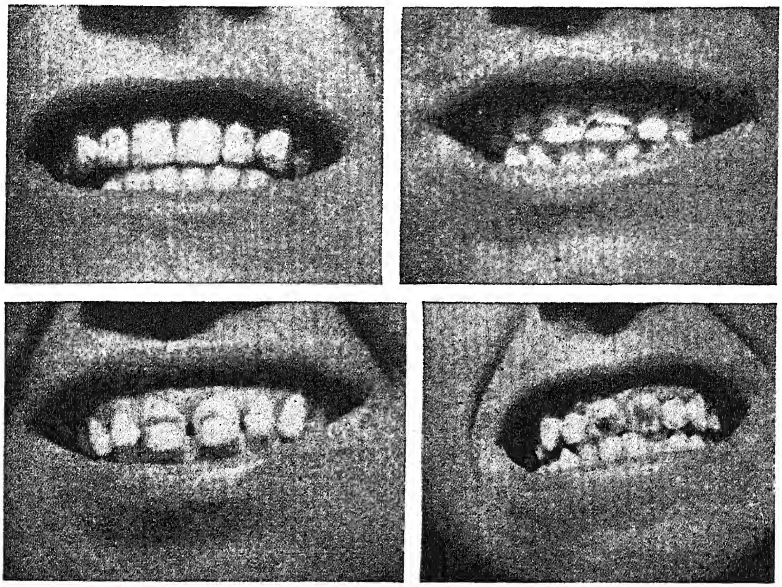

Fig. 102.—Typical cases of mottled enamel. (Bureau of Dental Health, Florida State Board of Health.)

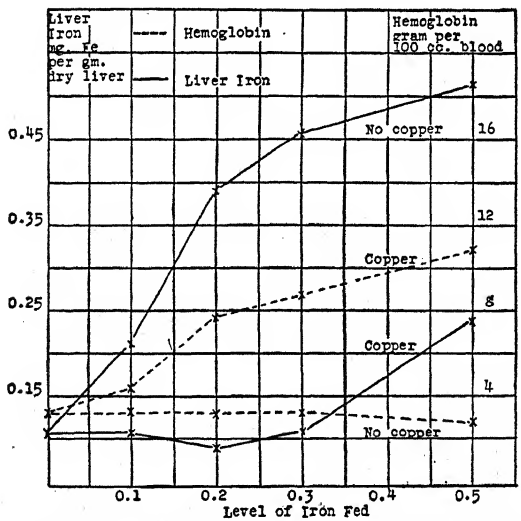

Fig. 103.—The effect of copper on the storage of iron in the liver and the hemoglobin content of the blood when graded levels of iron are fed. (Elvehjem and Sherman, *J. Biol. Chem.*, **98**, 315.)

The suggestion has been made that when the enamel contains appreciable quantities of calcium fluoride, it becomes more resistant to acid attacks.

Iron (See also Chap. 13).—As a result of recent work, discussions dealing with the utilization of iron must also include the element copper.

Most of the iron in the body is found as part of the hemoglobin molecule. Since a number of the oxidizing enzymes are heme compounds, they, also, would contain iron. Such a list would include cytochrome, Warburg's respiratory enzyme, catalase, and peroxidase. To this list is now added still another: Ferritin, an iron-protein complex which has been obtained from the liver, spleen and bone marrow. When radioactive iron is injected, it is stored in the liver as this ferritin. Apparently, ferritin acts as a storage for iron in the body.

It is now generally recognized that for the proper utilization of iron small quantities of copper are needed. Without the copper, the iron is assimilated but not converted into hemoglobin; such a conversion does take place in the presence of copper. This is very beautifully shown in the experiments by Elvehjem and Sherman (Fig. 103).*

TABLE 68.—AVAILABLE IRON DETERMINED BY DIPYRIDYL METHOD. (Elvehjem, Hart, and Sherman, *J Biol Chem.*, **103**, 61.)

Material.	Amount, gm.	Total Fe, mg.	Available Fe, mg.	Per cent available.
Wheat	3 0	0.15	0 07	47
Oats	3.0	0.21	0.12	57
"	4.4	0.30	0.17	57
Yeast	0 85	0.30	0.14	47
"	1 94	0.65	0.30	47
Ferric chloride		0.50	0.50	100
" pyrophosphate		0.50	0.50	100
" hypophosphite		0 50	0 50	100
" glutamate		0 50	0.44	88
Glutamic acid parahematin		0.50	0	0

It would seem, further, that "organic" iron—or rather iron of the "heme" type—cannot be used for the formation of hemoglobin; and that, therefore, whatever iron is present in the food in this form is not available. By the use of the dipyridyl reagent (α-α'-dipyridyl), which forms a colored product with the nonhematin iron only, a means of evaluating "available" iron is possible (Table 68).

Table 69 gives the amount of iron in some typical foods.

Anemias due to deficiencies in the amount of iron in the diet are very common. In a survey in Aberdeen, 41 per cent of infants (under two years of age) examined suffered from anemia. In investigations in East Africa on the effect of different diets, it was shown that in a population whose food consisted largely of cereals, 48 per cent of the boys were definitely anemic; whereas in an adjoining district, where the food included relatively large percentages of meat, blood, and milk, the anemic sufferers among a corresponding group numbered less than 12 per cent.

* Elvehjem suggests that cobalt also stimulates hemoglobin formation.

Anemias of this type—the typical "nutritional" anemia, due to a lack of "available" iron in the diet—can be treated with gratifying results by incorporating in the diet 25 mg. of iron pyrophosphate and 1 mg. of copper sulfate each day.

Using radioactive iron as "tracer," it can be shown that anemic animals (rats) eliminate less of the administered iron than normal ones. During a ten-day period, the normal animals retain about 30 per cent of the administered element, while the anemic animals retain 50 per cent.

The accumulation of the absorbed iron is greatest in the bone marrow, blood, spleen, liver and heart.

TABLE 69.—IRON IN TYPICAL FOOD MATERIALS (Sherman, *Chemistry of Food and Nutrition.* By permission of The Macmillan Company, Publishers.)

Food.	*Iron (in mg.) per 100 gm. fresh substance.*
Beans, dried	10.5
Egg yolk	8 6
Peas, dried	5 7
Wheat, entire grain	5.0
Oatmeal	4.8
Eggs	3.1
Beef	3.0
Prunes	2.8
Spinach	2.5
Beefsteak, medium fat	2.0
Cheese	1.3
Beans, string, fresh	1.1
Potatoes	1.1
White flour	1.0
Rice, polished	0.9
Beets	0.8
Carrots	0.6
Bananas	0.6
Turnips	0.5
Oranges	0.5
Tomatoes	0.4
Apples	0.3
Milk	0.2

It is possible that iron cannot be absorbed in the ferric state. If ferric iron is offered, whatever is absorbed is first reduced in the intestinal canal.

The iron in whole blood is given as 17 microgram per cc. (1 microgram = 1 μg. = 0.001 mg. = 1 gamma). Most of this iron is centered in the corpuscles. The amount of the element (in the inorganic form) in serum is about 1.68 μg. per cc.

It has been estimated that children need 0.6 mg. of iron ("available") and 0.1 mg. of copper per kilogram of body weight per day. Sherman is of the opinion that the daily needs for the adult are about 12 mg., and that the diet should also include 2 mg. of copper. During pregnancy, a daily diet including 20 mg. of iron is recommended.*

Contrary to the opinion held at one time that appreciable quantities of iron are excreted, particularly through the intestines, actually

* More recent figures by Whipple suggest that as little as 2–3 mg. suffices for the adult.

very little is excreted (under normal conditions) either by the kidney or the intestinal tract. The iron is conserved and used again and again as it helps to form hemoglobin which, in time, loses its iron (through conversion of the hemoglobin into bile pigments, etc.), paving the way for the freed iron to contribute once again to hemoglobin synthesis (and to the syntheses, in lesser amounts, of other iron-protein compounds such as cytochromes, etc.).

The bile pigments are excreted through the bile, but most of the protein and the iron of hemoglobin are utilized by the body for resynthesis.

In addition to emphasizing the importance of copper and the importance of supplying "available" iron, the "condition" of the subject has to be considered. Prolonged diarrhea often decreases the amount of iron absorbed, perhaps because the chyme does not remain long enough in the intestine. Patients suffering from achlorhydria, with deficient quantities of hydrochloric acid in the gastric juice, often suffer from anemia due to lack of utilizable iron. In one experiment, achlorhydric patients excreted 4.4 mg. of iron more than they ingested daily,

TABLE 70.—COPPER CONTENT OF BLOODS CONTAINING HEMOCYANIN. (Elvehjem, *Physiol. Rev.*, **15**, 472.)

Group.	Animal.	Copper.
		mg. per 100 cc.
Mollusca, Cephalopoda	Octopus vulgaris	23.5
	Sepia officinalis	23.7
Mollusca, Gastropoda	Helix pomatia	6.5–7.5
Crustacea, Decapoda	Astacus fluviatilis	7.0
	Palinurus vulgaris	9.5
	Homarus vulgaris	10.0
	Cancer pagurus	6.0
	Carcinus maenas	9.0
	Maia squinado	3.5
Crustacea, Stomatopoda	Squilla mantis	6.1

whereas "controls" (normal subjects) excreted slightly less than the amount consumed. The addition of hydrochloric acid to the diet of the patients suffering with achlorhydria did not affect their utilization of iron, although it is claimed that the addition of iron ammonium citrate was effective.

Copper (See Iron, p. 432).—The intimate association of copper with the metabolism of iron has made it impossible to separate the two elements. However, a few additional facts may be presented.

Copper is present in all living matter, both plant and animal. In plants, it seems to be associated in some way with the formation of chlorophyll. In animals, its association with hemoglobin formation has been stressed. The mechanism of this reaction is obscure.

Among naturally occurring organic substances, several contain copper. One of them, hemocyanin, a copper-protein complex, is found

in the blood of certain invertebrates (Table 70). In the crab, spider and snail, for example, the hemocyanin functions as an oxygen carrier similar to hemoglobin in man. Turacin is a pigment found in the feathers of the South African bird, turaco. Certain oxidizing enzymes, such as polyphenoloxidase (p. 399), laccase (p. 399) and tyrosinase are copper-protein complexes.

Other copper compounds were discovered by Keilin and Mann, who isolated a copper-protein compound from the red blood corpuscles of

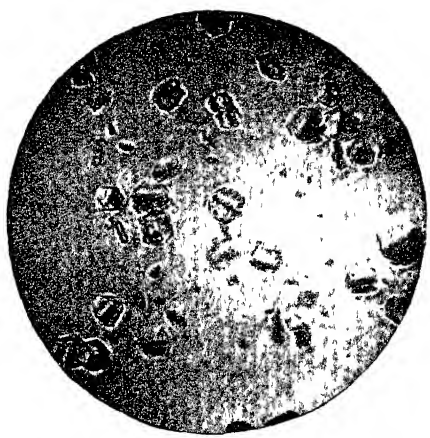

Fig. 104.—Crystals of hemocuprein containing 0 34 per cent Cu from red blood corpuscles of an ox. (Keilin and Mann, *Proc. R. S.* (London), Series B, **126**, 303)

mammals, to which they gave the name *hemocuprein* (Fig. 104). They have isolated another copper-protein compound, *hepatocuprein*, from the liver. "It is certain," they write, "that some of the copper supplied . . . as food is utilized for building up hemo- and hepatocuprein. The formation of these compounds may therefore represent one of the

Table 71.—The Copper Content of Foods (Average Figures).

Substance (per kg.).	*Copper (in mg.).*
Liver	44.1
Nuts	11.6
Legumes	9.0
Cereals	4.7
Fruits	4.2
Poultry	3 0
Fish	2 5
Green legumes	1.7
Leafy vegetables	1.2

steps in copper metabolism and may directly or indirectly be responsible for some of the physiological effects which are rightly ascribed to copper."

The copper content of several foods is summarized by Rose (from data by Elvehjem) (Table 71).

Cow's milk contains from 0.09 to 0.17 mg. of copper per liter. Nelson has isolated a copper-protein compound from milk.

It has been estimated that the daily needs of copper are in the

neighborhood of 2 mg. per day. Much of its excretion is via the bowels In a particular experiment, on an intake of 2 17 mg., 1.22 was eliminated with the feces, and 0 27, with the urine. The adult body is said to contain from 100 to 150 mg. of the element.

The claim has been made that copper plays some rôle in skin and hair pigmentation. It is said that the element accelerates the oxidation of "dopa," 3,4-dihydroxyphenylalanine, by "dopa oxidase," an enzyme present in the skin.

Some experiments by Schultze have shown that the anemia in the rat accompanying a severe copper deficiency is further accompanied by a decrease in the cytochrome oxidase activity of the bone marrow—a loss which is offset when copper is fed again. The bone marrow, it should be emphasized, is one of the chief centers of hematopoiesis (the formation of blood from food products), and it would seem as if copper plays an essential part in this reaction.

Aluminum.—This element is a constituent of plant and animal tissues. Various foods contain aluminum; and a certain amount of the element finds its way into the body from the cooking utensils employed. The available evidence is that the small amount of aluminum which may be absorbed as a result of using aluminum utensils is not harmful.

Whether the element is essential or not cannot be answered at present. However, it has been shown that feeding rats with relatively large amounts of aluminum salts produces severe rickets, due to the fact that such salts retard the assimilation of phosphates.

Aluminum is poorly absorbed; more than 70 per cent is found in the feces.

Cobalt.—Experiments which suggest the essential nature of the element have been published in connection with a disease of cattle and sheep in Australia, known as "enzootic marasmus," and characterized by progressive emaciation and anemia, followed by death. The evidence points strongly to insufficient cobalt in the diet. As little as 0.3 to 1 mg. of cobalt given daily to cattle is sufficient for normal growth and health in an otherwise affected area.

Coast disease, which afflicts sheep in South Australia, is somewhat similar to enzootic marasmus, and may be cured by a mixture of cobalt and copper.

Using radioactive cobalt, it has been determined that most of the element is quickly eliminated from the body; which would indicate that the requirement for cobalt by the body is very small.

The element is eliminated almost completely via the kidneys. This is in contrast to manganese, which is excreted almost exclusively in the feces, and in contrast to iron, which may be excreted both through the large intestine and through the kidneys.

Cobalt produces a polycythemia (an excess in the number of red corpuscles in the blood) when fed or injected. As little as 0.04 to 0.05 mg. in the entire body (of the rat) is enough to develop this polycythemia. Apparently, if cobalt is an essential element, the amount needed by the body certainly does not exceed "traces."

Manganese.—This element is found in plant and animal tissues and seems to belong to the "essential" group. When present in deficient amounts there is a marked retardation of growth in the rat. In the female, estrous cycles are irregular, and in the male one finds testicular degeneration and sterility due to absence of spermatozoa.

It was at one time believed that manganese, like copper, is of importance in the proper utilization of iron in the body; but this view has met with much opposition.

Bertrand has shown that manganese is a necessary element for the development of the young plant; and he has even suggested that its particular function is to cooperate in the action of the oxidases in the plant; but this view has been questioned.

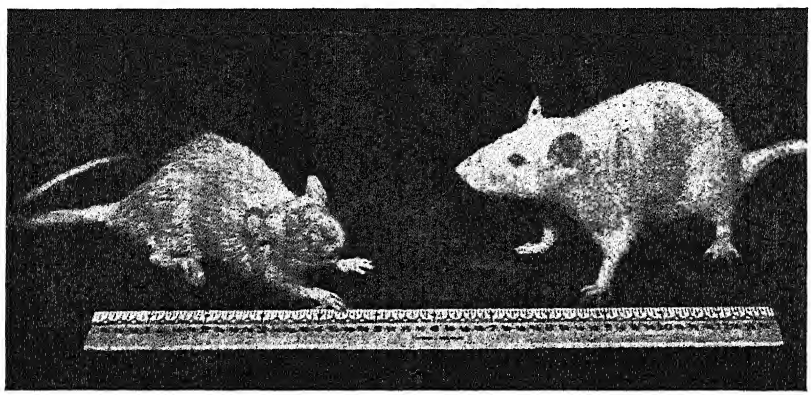

Fig. 105.—Manganese-deficient rat with normal litter mate. Animals are 6 months old, ♀ ♀, and weigh 130 grams and 230 grams respectively. (Wachtel, Elvehjem and Hart, *Am. J. Physiol.*, **140**, 72 (1943).)

Manganese deficiencies in the diet have been reported to cause testicular degeneration and impaired estrous cycle in mice, lameness in pigs, sterility in cows, and perosis (p. 147) in chicks.

Elvehjem and Hart, in some extensive experiments on rats, showed that on a synthetic diet which included but 5 γ of manganese per day (γ = gamma = 0.001 mg.), the growth of the animals was impaired (Fig. 105). The deficient rats had poorer bone formation, and their serum phosphatase was increased two- to three-fold.

The kidney and the liver are the chief storage places for the element in the body. In 100 gm. of tissue, we find 0.17 mg. in the liver and 0.087 mg. in the kidney. The manganese content of blood varies from 0.004 to 0.020 mg. per 100 cc. The element is very slowly absorbed, most of it being found in the feces.

It was pointed out under "cobalt" (p. 436) that manganese is very largely excreted with the feces. Greenberg, using the radioactive isotope of manganese, makes it clear that the bile plays an important rôle in the intestinal excretion of the element, for some 50 to 75 per cent of it is carried by the bile.

The amount of manganese in foods varies considerably. In terms

of kilogram of dry material, those having from 100 to 200 mg. are beet tops, blueberries, lettuce, pineapple, and wheat bran; those from 35 to 100 mg , beets, blackberries, spinach, whole grain wheat. Fruits, as a rule, have less than 15 mg. per kilogram, and round of beef and the fish so far examined have little or none.

Zinc.—The weight of evidence is in favor of the view that zinc is an "essential" element. Todd, Elvehjem, and Hart kept young rats in monel-metal cages and fed them a highly purified ration which included all of the known vitamins and essential mineral elements, together with 2 cc. of milk. The only probable deficiency of this diet was its low content of zinc—1.6 mg. per kilo of the food offered. These animals were compared with controls which received more zinc; the former were inferior to the latter in rate of growth and maximum weight attained. There is also, on this low zinc diet, some interference with the development of a normal fur coat.

Scott has shown that zinc is a constituent of crystalline insulin (p. 493); but the full significance of this discovery is not yet apparent. Zinc is also a constituent of the enzyme, carbonic anhydrase (p. 306).

The pancreas (of beef, calf, sheep, and hog) contains from 19 to 40 mg. of zinc per kilogram of gland.

It has been estimated that the average daily diet contains 12 to 20 mg. of the element. Much of the excretion is by the intestinal tract Radioactive zinc accumulates in the mucosa of the intestine and in the pancreas and the liver.

Sulfur.—Most of the sulfur is found in the protein molecule and is therefore part of an "organic" molecule. In fact, the metabolism of sulfur, just like the metabolism of nitrogen, is very intimately associated with the metabolism of protein itself.

The sulfur of the protein is centered in the groups containing cystine and methionine. The "essential" nature of the latter, and the metabolism of both amino acids, have already been discussed (see p. 367).

Sulfur is largely oxidized to sulfate in the body and excreted as "inorganic" and "ethereal" sulfates, both of which will be discussed under "Urine" (p. 469).

Besides cystine and methionine, organic compounds of sulfur found in the body are glutathione, insulin, thiamine, ergothionine, taurocholic acid, sulfocyanide, "ethereal" sulfates (esters of phenols and sulfuric acid), chondroitin sulfuric acid (in cartilage, etc., p. 450) and melanins (pigments of the body). Small quantities of inorganic sulfates (mainly sodium and potassium) are found in the blood and various tissues of the body.

The average percentage of sulfur in various foods is 1 per cent.

The use of radioactive sulfur (obtained by bombarding sulfur with fast-moving deuterons from a cyclotron) in studying sulfur metabolism, has been introduced. The radioactive sulfur was oxidized to sulfuric acid and then converted to sodium sulfate, the form in which it was fed. The urinary sulfur was converted to barium sulfate and its radioactivity measured by the use of a suitable electroscope. Fifteen

per cent of the radioactive material appeared in the urine in the first nine-hour period, and 32 per cent in the second fifteen-hour period. No radioactive material was excreted on the following two days. When added sulfate enters the tissues, there is apparently an exchange with the sulfate already there, and its excretion is delayed.

Selenium.—In certain localities in this country—in South Dakota and Wyoming—plants may contain enough selenium to produce toxic effects when fed to animals. What is known as "alkali disease" or

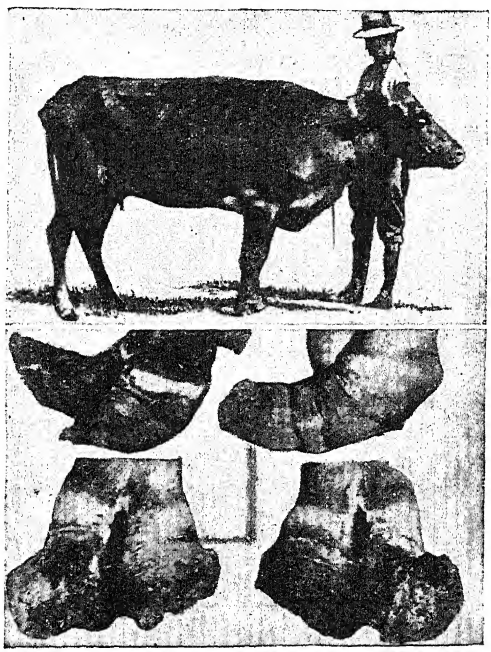

Fig. 106.—The chronic form of selenium poisoning of cattle. Upper, Cow affected with alkali disease. Lower, Deformed hoofs of cattle. (U. S. Depart. of Agric.)

"blind staggers" among the natives, is apparently the result of poisoning due to this element (Fig. 106). Soils containing more than 0.5 part per million of selenium are regarded as "potentially dangerous."

"Selenium," writes Trelease, "appears to be needed by only a few plants; it is tolerated by other plants; and, as far as we know, it is toxic but never beneficial to animals or man."

Using selenite containing radioselenium, with rats as subjects, it has been possible to show that most of the selenium is excreted by way of the kidney.

WATER

Of all compounds in the body, water is the most abundant. It constitutes more than 60 per cent of the total weight of the body. We find it in cellular and vascular spaces; and small portions are also deposited in conjunction with protein and carbohydrate. The storage of fat, however, is accompanied by little or no water.

Water is present in every tissue, but the amounts vary considerably. Table 72, the data for which were collected by Rowntree, gives some figures.

TABLE 72.—NORMAL WATER CONTENT OF THE TISSUES (IN PERCENTAGES)

Tissue	Matthews	Long	Robertson
Saliva	..	99.5	
Cerebrospinal fluid	..	99.0	
Vitreous humor	..	98.5	
Embryonic brain	91		
Milk	..	88.0	
Brain (gray matter)	84	86.0	82–85
Kidney	78	83.0	
Thyroid	..	....	77–82
Thymus	77	....	81
Suprarenal	80		
Blood	..	79.0	
Pancreas	..	78.0	
Muscle	73	75.0	75–78
Spleen	..	76.0	
Liver	76	70.0	
Skin	..	72.0	
Brain (white matter)	68	....	68–70
Tendon	..	....	56–68
Cartilage	67		
Elastic tissue	..	50.0	
Bones	..	50.0	Vertebrae and ribs 16–44 Extremities and skull 14–22
Fat	6–10	20.0	
Dentin	10	10 0	

Function.—A very large percentage of the water is of the utmost importance physiologically—as solvent, as a "carrier" in transporting foods to tissues, and wastes from tissues; as a regulator of body temperature, etc.

Heat is gained by the oxidation of foodstuffs in the body. Heat is lost through the following channels (in percentages): urine and feces,

1.8; warming of expired air, 3.5; vaporization from lungs, 7.2; evaporation from skin, 14.2; radiation and conduction from skin, 73.0.

Amount Needed.—The needs of the body for water are met in two ways: by direct intake, and by the oxidation of the foodstuffs in the body. By "direct intake" we mean water as such and water present in foods. Roughly speaking, on a diet equivalent to 3000 calories, probably 2000 cc. of water is derived from the water in foods and from the products of the oxidation of foodstuffs in the body. Since the requirement may be some 3000 cc. per day, another liter of water must be supplied.

The amount of water liberated as a result of the oxidation of foodstuffs is approximately 10 to 14 gm. per 100 Calories. For example, 100 gm. of fat, when oxidized, produces 107 cc. of water. The 100 gm. of fat is equivalent to 930 Calories, which means that fat equivalent to 100 Calories will produce 11.5 cc. of water. Similarly, carbohydrate and protein equivalent to 100 Calories each will produce 13.5 and 10.1 cc. of water, respectively.

Regulation.—Since the amount of water in the body tends to vary but little, the regulation of the water balance in the body is a very important matter. Not only is water taken into the system in the manner already referred to, but it also leaves the body through several channels—urine, sweat, expired air, feces, and, in much smaller quantities, tears. The regulatory mechanism balancing gain and loss is still obscure; but it is believed that one controlling factor is the antidiuretic hormone present in the posterior lobe of the pituitary. An extract can be obtained from the pituitary which will stop the excessive output of urine (polyuria) in diabetes insipidus. It is possible that, under normal conditions, this hormone controls the extent of reabsorption of water through the kidney tubules.

Swingle has maintained that an important function of cortin, the hormone of the adrenal cortex (p. 498), is to regulate the volume of blood. He claims that in the absence of cortin, water passes from the blood to the tissues, thereby reducing the blood volume; a result which proves fatal.

DuBois gives the following figures illustrating intake and output of water (the subject was a nephritic patient):

1. *Water intake*

Drinking water	300 cc.
Water in coffee, milk, soup	580 cc.
Water in "solid" foods	720 cc.
Water from oxidation of 100 gm. protein	41 cc.
Water from oxidation of 100 gm. fat	118 cc.
Water from oxidation of 244 gm. carbohydrate	135 cc.
Total	1894 cc.

2. *Water output*

In urine	750 cc.
In feces	180 cc.
Vaporized through skin	550 cc.
Vaporized through lungs	350 cc.
Total	1830 cc.

The water loss through the lungs is quite constant, but the loss through the sweat glands is associated with their function as regulators of body heat.

Dehydration.—This term is applied to the loss of fluid from the body. When water is lost, electrolytes (Na,K, etc.) are also lost. If insufficient water is consumed, some electrolyte must be eliminated so as to maintain the ionic concentrations of the body fluids. From this it also follows that the removal or loss of electrolyte requires the removal of some water

The removal of sweat and the loss of gastro-intestinal secretions removes not only water but electrolyte. "Dehydration," writes Gamble, "is an incomplete term since it does not indicate the accompanying loss of electrolyte." The treatment of "dehydration," then, means not only replacing the water lost but also the electrolyte lost.

As showing fine internal adjustments, some ten per cent of the body's weight may be lost due to depriving the individual of water without any appreciable reduction in blood volume.

Shock.—Shock in battle, usually produced by burns, wounds and severe hemorrhage, causes disturbances in body fluids: (For treatment, see under Blood, p. 265).

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CHAPTER 22

CHEMISTRY OF THE TISSUES

An examination of the head, the trunk, and the limbs of the body reveals certain similar "types" of substance in each of them; such as bone, cartilage, muscle, nerve. These similar "types," composed as they are of groups of similar cells, are called *tissues*. In this chapter we shall discuss the chemical composition of these tissues.

MUSCULAR TISSUE

This tissue consists of two varieties: striated muscle (voluntary), such as is found in the skeletal muscles of the body; and nonstriated, or smooth, muscle (involuntary), such as we see in the walls of the viscera, bladder, skin, arteries, veins.

The pronounced characteristic of muscular tissue is its ability to contract. The actual contraction is due to contractile fibers present in the cytoplasm of the cell.

A chemical examination of muscle—and most of the work has been done on striated muscle—shows it to be composed of 75 per cent of water and 25 per cent of solids. Twenty per cent of the solids consist of proteins, of which myosin, a globulin, is the most important. The remainder (5 per cent of the solids) includes carbohydrate, inorganic salts, "extractives" (p. 447), etc.

Table 73.—Properties of Muscle Proteins. (Needham, *Ann. Rev. Biochem*, 6, 395.)

		Albumins.		Globulins.	
		Myogen.	Myo-albumin.	Myosin.	Globulin x.
Isoelectric point.		6.7	3.3	5.5	5.2
Solubility of native protein in	H_2O	+	+	−	−
	Salts	+	+	+	+
	HCl	+	+	+	+
Solubility of denatured protein in	H_2O	−	−		−
	Salts	−	−		−
	HCl	+	−		+

Muscle Proteins.—Four proteins have been identified: myosin, 68 per cent; globulin x, 21 per cent; myogen, 10 per cent; and myoalbumin, 1 per cent (Table 73).

A liquid known as "muscle plasma" can be expressed out of muscle. This plasma from muscle can coagulate in a manner somewhat similar to the plasma obtained from blood. The changes are attributed to changes in the muscle proteins, particularly the myosin; but what these changes are is not clear (see references at the end of the chapter). Soon after death, in the state of rigor mortis, the muscle loses irritability and becomes rigid; these changes are attributed to the coagulation of the muscle proteins

Mirsky and Anson have shown that, when proteins are coagulated by denaturing agents (heat, acid, etc.), there is a change in the sulfhydryl and disulfide groups. When myosin becomes insoluble in rigor, however, there is no detectable change in its sulfhydryl groups. Mirsky finds that in the coagulation of myosin caused by dehydration, loss of solubility occurs without a change of groups; and this method of coagulation *in vitro* resembles, therefore, the coagulation of muscle in the cell (during rigor).

Myosin and Adenosinetriphosphatase.—An enzyme, adenosinetriphosphatase, hydrolyzes adenosinetriphosphate (ATP) to form adenosinediphosphate (ADP) and inorganic phosphate (p. 320), releasing a relatively large amount of free energy. This enzymatic reaction is considered to be the source of the energy needed for muscular contraction.

The discovery associating the enzyme (adenosinetriphosphatase) with myosin—the myosin which is probably responsible for the contractile and elastic properties of muscle—we owe to Engelhardt and Lyubimova. They believe the enzyme* to be myosin itself, and this view is meeting with general acceptance. As evidence, it is pointed out that the enzyme properties of myosin are unchanged throughout a series of mild reactions—reprecipitation, dilution and salting out. No protein fraction other than myosin itself shows these enzyme properties to anywhere near such an extent. Any reaction, however mild, which denatures myosin also destroys the properties of the enzyme.

The myosin, acting as an enzyme, acts only on certain triphosphates; besides ATP, it also acts on inosinetriphosphate and possibly inorganic triphosphate.

The enzyme activity is enhanced by calcium ions and, to a lesser extent, by manganese; whereas a number of investigators claim that magnesium has an inhibitory effect.

The myosin (enzyme) activity shows an optimum reaction at a pH of 9.0.

There is experimental evidence that ATP is involved in changes in the mechanical properties of muscle. "The Russian workers (Engelhardt, etc.)," write Green and Colowick, "find that when myosin threads, prepared by extrusion of a salt solution of myosin in water, are subjected to a given load, their extensibility is increased by the addition of low concentrations (0.0025*M*) of ATP . . . The effect is immediate and can be reversed when the thread is returned to water

* Polis and Meyerhof, *J. Biol. Chem.*, **163**, 339 (1946), claim to have partially separated adenosinetriphosphatase from myosin.

or dilute salt solution. Conditions which abolish the enzyme activity of myosin also abolish the effect of ATP on the extensibility of the myosin fibril."

The evidence also points to the fact that the first reaction between myosin and ATP—prior to hydrolysis—is a combination of the two, the combination of enzyme-substrate so familiar in enzyme reactions (see p. 105).

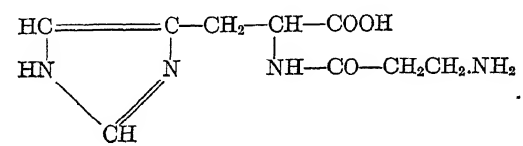
An analysis of the amino acids in myosin (rabbit) give the following figures (per cent): cystine 1.39; methionine 3.4; serine 3.57; threonine, 3 81; tyrosine, 3.4; aspartic acid, 8.9; glutamic acid, 22.1; arginine, 7 0; lysine 10.3; histidine, 1.7; tryptophan, 0.82; glycine, 1.9; alanine, 5.1.

Muscle Phosphorylase.—We have already discussed this enzyme under carbohydrate metabolism (p. 322). It will be recalled that it is the enzyme which catalyzes the reaction: glucose-1-phosphate $\rightleftarrows$ polysaccharide + inorganic phosphate.

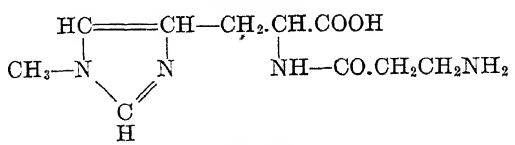
"Extractives."—What are known as "extractives" of muscle are substances which can be extracted by means of water, alcohol, or ether. They are divided into two groups: those which contain nitrogen and those which do not contain the element.

"Extractives" Containing Nitrogen.—Many of these substances have already been discussed. They include creatine, phosphocreatine, purine bases, uric acid, adenylic acid, carnosine, anserine, etc. The "flavor" of meat is attributed, very largely, to these "extractives."

Carnosine (a dipeptide of histidine and β-alanine) and anserine (a β-alanyl derivative of methylhistidine) have the following structures:

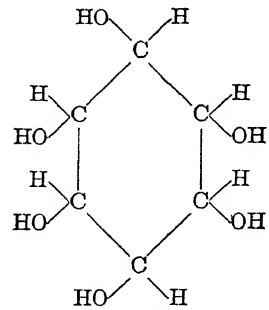


Carnosine.



Anserine.

"Extractives" Containing No Nitrogen.—Most of these substances have also been discussed. They include glycogen, hexosephosphates, lactic acid, etc. A peculiar substance which is present is *inositol*, a hexahydroxycyclohexane. This substance, in fact, is present in many tissues and has been identified with one of the vitamins (p. 170). In plants it is found in combination with phosphoric acid. *Phytin* is the calcium-magnesium salt of such a combination.



Inositol.

Inorganic Salts.—Potassium and phosphate ions are the most numerous. Other elements are sodium, calcium, magnesium, iron, and chlorine.

NERVOUS TISSUE (See Chap. 25)

The nervous tissue includes the brain, spinal cord, peripheral nerves, ganglia, and plexuses. Its importance lies in its ability to respond to stimuli and to conduct impulses.

A chemical analysis reveals the presence of proteins, lipids, inorganic salts, etc. Of the total solids—which may amount to some 17 per cent in the case of the gray matter of the brain—approximately one-half consists of proteins. These include a nucleoprotein, two globulins, and an albuminoid called *neurokeratin.*

The lipids have already been discussed (Chap. 3). They include lecithin, cephalin, sphingomyelin, cerebrosides, cholesterol, etc.

EPITHELIAL TISSUE

The epithelial tissue is found in the covering of the surface of the body (the skin), in the lining of the respiratory tract, as an essential part of glandular organs, etc.

Keratin.—The characteristic substance present in this tissue is the albuminoid keratin. Among proteins, it is the most resistant to chemical action. It is insoluble in any of the solvents which dissolve other proteins and is not attacked by gastric or pancreatic juice. This protein is further characterized by its high sulfur content, of which most is in the form of cystine. Human hair, for example, contains from 16 to 21 per cent of this amino acid. On acid hydrolysis, keratin yields the basic amino acids, histidine, lysine, and arginine in the molecular ration of 1 : 4 : 12.

TABLE 74.—COMPOSITION OF EUKERATIN OF HUMAN HAIR. (Block, *J. Biol. Chem*, **128**, 181.)

	Per cent.
Histidine	0 6
Lysine	2 5
Arginine	8 0
Cystine	15 5
Tyrosine	3 0
Tryptophan	0 7
Phenylalanine	2.6
Glycine	4.3

Block divides the keratins into two groups: eukeratins and pseudokeratins. Both varieties are insoluble in aqueous and organic solvents, and both are practically not attacked by proteolytic enzymes; but the eukeratins alone show the constant histidine-lysine-arginine ratio.

The composition of eukeratin obtained from hair is given in Table 74.

Though normally so resistant to chemical and enzymic action, by exhaustive grinding keratin becomes more digestible. For example, if wool fibers (rich in keratin) are first ground in a ball mill, they can be digested by both pepsin and trypsin. Significantly enough, after grinding, these wool fibers if extracted with water show larger quantities of nitrogen and sulfur constituents in solution than before grinding. Similar results are obtained with keratins from human hair, turkey feathers, duck feathers, chicken feathers and porcupine quills.

That such mechanical grinding, aside from increasing surface area and so enabling the proteolytic enzymes to come into more intimate contact with substrate, is accompanied by some chemical change is made probable by the fact that after the operation, there is a decrease in cystine sulfur, and one-half to one-fourth of the water-soluble sulfur is in the form of inorganic sulfates, a change which suggests some oxidation.

Suggestive, too, are preliminary experiments with animals which lead to the conclusion that such finely ground keratin can be utilized to some degree as the protein constituent in the diet of animals. Supplementing the diet with tryptophan, methionine and histidine—amino acids present in very small quantities—improved the condition of the animals. To convert what has always been considered a purely waste product from the point of view of food—keratin—into a useful animal "feed" would be an important practical achievement.

Male hair, it seems, contains more cystine than female hair; and dark hair contains more cystine than light hair.

According to Edwards and Duntley, the color of normal skin is due to several pigments: melanin (p. 366), a closely-related substance which the authors call "melanoid," carotene, reduced and oxy-hemoglobin. "Our studies confirm the idea that the colored races owe their characteristic color only to variations in the amount of melanin present. . . . No pigments other than those found in the whites are encountered in the dark races. . . ."

Human red hair yields a red iron pigment of unknown constitution. This pigment cannot be obtained from human hair unless it is bright red in color.

CONNECTIVE TISSUE

The connective tissue, including cartilage and bone, acts as supporting and binding material, and is, among the tissues, the most widely distributed. Under this heading we shall discuss briefly white fibrous tissue, yellow elastic tissue, cartilage, bone, and teeth.

White Fibrous Tissue.—The main organic constituent is the albuminoid *collagen*, which, like keratin, is chemically resistant, but not to

the same degree. The collagen is fairly well digested by pepsin, but only slightly by trypsin Preliminary treatment with alkali makes the albuminoid more digestible by trypsin. It contains much less sulfur (in the form of cystine or methionine) than does keratin. By boiling with water, the collagen is converted to *gelatin*, a much more easily digestible protein. The chemical nature of this interconversion is still not clear; the tendency is to regard the change in the nature of an intramolecular rearrangement.

An analysis by Gies of the *tendo Achillis* of the ox—an example of white fibrous tissue—reveals that the solids constitute from 35 to 40 per cent, only a fraction of a per cent of which is inorganic matter. The organic matter is composed of the following (in per cent): collagen, 31.6; elastin, 1.6; mucoid, 1.2; fatty substance, 1.0; etc.

The elastin is referred to in the next section. The mucoid (tendomucoid) resembles the mucin of the saliva; which means that it is a glucoprotein.

Yellow Elastic Tissue.—According to Gies, the analysis of *ligamentum nuchae*—an example of yellow elastic tissue—yields some 40 per cent of solids, composed of the following (in per cent): elastin, 31.6; collagen, 7.2; fatty substance, 1.1; mucoid, 0.5; inorganic matter, 0.4; etc.

The *elastin*, like keratin and like collagen, belongs to the class of proteins known as albuminoids; which means that it is, relatively speaking, a chemically resistant protein. In general, it resembles collagen in its properties, although, unlike the latter, it is not changed to gelatin when boiled with water.

Elastin is slowly digested by pepsin and trypsin. Table 75 gives an analysis of elastin.

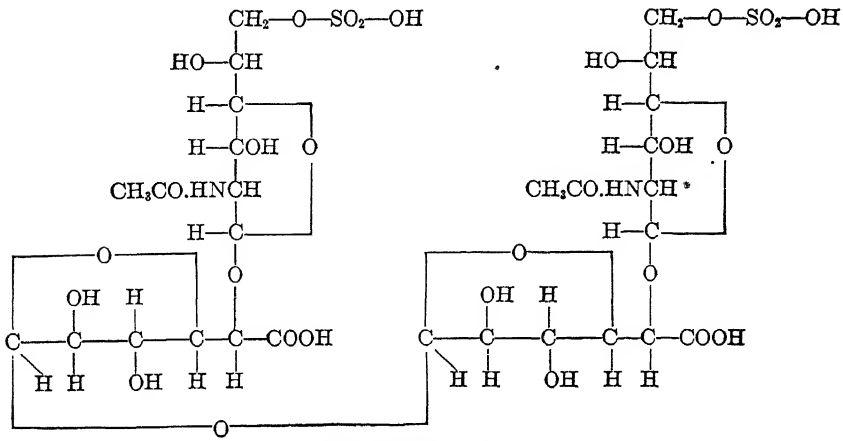
TABLE 75.—AMINO ACIDS IN ELASTIN. (Stein and Miller, Jr., *J. Biol. Chem.*, **125**, 599.)

Amino acid.	Per cent by weight.
Glycine	29 4
Alanine	None found
Valine	13 5
Aspartic acid	0 0
Arginine	1 0
Lysine	None found
Histidine	0 0
Cystine	0 23
Methionine	0 38
Tyrosine	1.6
Tryptophan	0 0
Proline	15 2
Hydroxyproline	2 0
"Leucine fraction"	30 0
Phenylalanine*	3 34
Amide N	0 04
Total	85 0

* From data of Kapeller-Adler.

Cartilage.—This rather tough and firm material, popularly known as "gristle," contains an organic sulfur compound called *chondromucoid*,

a glycoprotein, which, on hydrolysis, yields chondroitin sulfate, a combination of sulfuric acid, glucuronic acid, chondrosamine (2-amino-galactose), and acetic acid:



Chondroitin sulfate.

This condroitin sulfate is also found in osseo- and tendomucoids; and, according to Jorpes, it is the essential constituent of heparin, an anticoagulating material (p. 270). The mucin of saliva also contains a somewhat similar substance, known as mucoitin sulfate; but the latter contains glucosamine rather than 2-aminogalactose.

A chondroalbuminoid similar to elastin, and collagen, constitute the other two proteins of cartilage.

BONE* (Osseous Tissue)

The organic matrix is similar to that found in cartilage and connective tissues in general. We find collagen, a glycoprotein and an osseoalbuminoid. The normal, mature bone contains nearly one half its weight of water, and sometimes as much as 24 per cent of fat. Using the dry, fat-free material, some 30 to 40 per cent of this substance is organic in nature. The chief inorganic constituents are calcium, phosphate, and carbonate (Table 76).

TABLE 76.—COMPOSITION OF BONE. (Morgulis, *J. Biol. Chem.*, **93**, 455; Hammet, *Ibid*, **64**, 693.)

Animal.	Condition.	Calcium.	Magnesium.	Phosphorus.	CO_2.
		In per cent of total bone ash			
Dog	Normal	35.7	0.46	15.8	5.6
Cow	Normal	36.1	0.74	16.4	4.6
Rabbit	Normal	36.3	0.53	16.0	5.7
Hen	Normal	37.2	0.51	16.4	5.5
Rat	Normal	37.5	0.85	18.5	

* Compare this section with the section devoted to teeth (p. 453).

Among the organic constituents is citric acid, a substance which was overlooked for a long time, probably because in the ordinary analysis of bone as "bone ash" the citrate will have been converted into carbonate and recorded as such.

Dickens has found citric acid—probably as the calcium salt—in bone to the extent of about one per cent. It may be of use to the organism in two ways: as a reserve supply of citrate in the course of the metabolism of carbohydrate (p. 325); and as a solvent for the inorganic substances in bone (such as calcium carbonate and calcium phosphate),—probably as a preliminary step in the active metabolism of bone (see below).

Inorganic Salts.—The principal inorganic constituents of bone are calcium, phosphate and carbonate, with lesser quantities of magnesium and sodium. The x-ray diffraction pattern of bone is similar to that of the mineral apatite, which is sometimes written as $Ca_{10}(PO_4)_6F_2$. Substitutions of $(OH)^-$ for F^- and Mg^{+2} for Ca^{+2} occur without any particular change in the diffraction pattern.

Various formulas for the phosphate substance of bone have been suggested, but none has been found convincing. For example, the suggestion has been made that the substance is a complex carbonate apatite, $Ca_{10}CO_3(PO_4)_6$; or that it is a hydroxyapatite, $Ca_{10}(OH)_2(PO_4)_6$; or that it is some multiple apatite, $3Ca_3(PO_4)_2 \cdot CaX$, where $X = O$, Cl_2, SO_4, CO_3, F_2, etc.

In any case, the composition of the inorganic constituents of bone is by no means constant. Changes occur with age, in rickets, in acidosis and alkalosis, as a result of change in diet, etc. If, for the sake of convenience, we confine ourselves for the moment to a basic formula which has often been used, $CaCO_3.nCa_3(PO_4)_2$, the most frequent changes occur in the percentage of carbonate.

Sobel and Kramer have shown that the calcium and phosphorus content, and the vitamin D content of the diet influence the CO_3:Ca ratio of bone.

Metabolism.—That there is active metabolism in bone was shown by Hevesy who used labelled (radioactive) phosphorus and found that some of the phosphorus atoms of the mineral constituents of the bone exchange rapidly with those present in the plasma. Within 50 days, 29 per cent of the mineral constituents of the femur and tibia epiphyses were found to be renewed.

Factors Affecting Calcification.—(*a*) *The Calcium and Phosphorus of the Blood.*—Studies in this connection are confined almost wholly to rickets (p. 178). In this disease the bone fails to calcify because of a lowering in the serum concentration of calcium, or phosphate, or both.

Approximately one-half of the calcium is in a nondialyzable form, presumably bound to protein; most of the diffusible calcium is ionizable.

From the work of Robison and others, it seems clear that an important factor in the process of calcification is the presence of the enzyme *phosphatase*. This enzyme, present in bones, teeth, and ossifying cartilage of young animals, hydrolyzes hexosemonophosphoric ester and glycerophosphoric ester, liberating inorganic phosphate. A rachitic

bone, cut longitudinally and placed in a solution of calcium hexose-monophosphate or calcium glycerophosphate, absorbs calcium phosphate and deposits the salt in the zones prepared for calcification. The conclusion has been drawn from this work that the bone phosphatase acts on the organic phosphorus ester liberating inorganic phosphate which, in turn, affects the product of the calcium and phosphate ions in solution to such a degree that the solubility product is exceeded, and the excess calcium phosphate is deposited.

(*b*) *The Calcium and Phosphorus in Food.*—According to Sherman, the diet should include the following daily amounts (in grams):

	Ca.	*P.*
Children (3–13 years of age)	1.0	1.16–1.46
Adults	0.45	0.88

These are minimum requirements.

(*c*) *Vitamin D* (see p. 178).

(*d*) *Reaction of the Intestinal Tract.*—Dogs fed a normal diet show a *p*H in the small intestine varying from 5.7 to 6.6. When these animals are fed a rickets-producing diet, the *p*H of the intestine is changed to 6.4–7.4. The addition of cod liver oil, or irradiation of the animal, lowers the *p*H more to the acid side (Grayzel and Miller) Apparently, in a more acid medium, the calcium salts are more soluble, and, therefore, more easily absorbed.

(*e*) *The Parathyroid Hormone.*—The relationship of calcium to the parathyroid hormone is discussed elsewhere (p. 483).

TEETH

The tooth consists of three calcified parts: the dentin, the chief substance of the tooth surrounding the tooth-pulp; the cementum, covering the root of the tooth; and the enamel, the hardest of the three, covering the dentin (Fig. 107). These three, the dentin, the cementum, and the enamel, contain both inorganic and organic matter.

Organic Matter.—The percentages of organic matter are dentin, 25; cementum, 30; enamel, 1. The main constituent in enamel is keratin, with smaller quantities of cholesterol and phospholipids. In dentin we find mainly collagen, with less elastin and small quantities of lipids. Collagen is found in cementum.

The Inorganic Matter.—The inorganic matter (in percentage) is approximately 99.5 for enamel, 77 for dentin, and 70 for cementum. The following figures give the results of analysis of human enamel and dentin (Karshan), and for comparison, an analysis of human bone (Gabriel).

	Enamel.	*Dentin.*	*Bone.*
Calcium	35.77	26.5	23.84
Magnesium	0.27	0.79	0.30
Phosphorus	17.43	12.7	10.41
Carbon dioxide (from carbonate)	2.97	3 06	3.81

Enamel also contains (in per cent) 0.25 of sodium, 0.05 of potassium, 0.3 of chlorine, 0.0112 of fluorine and 0.0218 of iron; and dentin

(in per cent) 0.19 of sodium, 0.07 of potassium, 0.0 of chlorine, 0.0204 of fluorine and 0.0072 of iron.

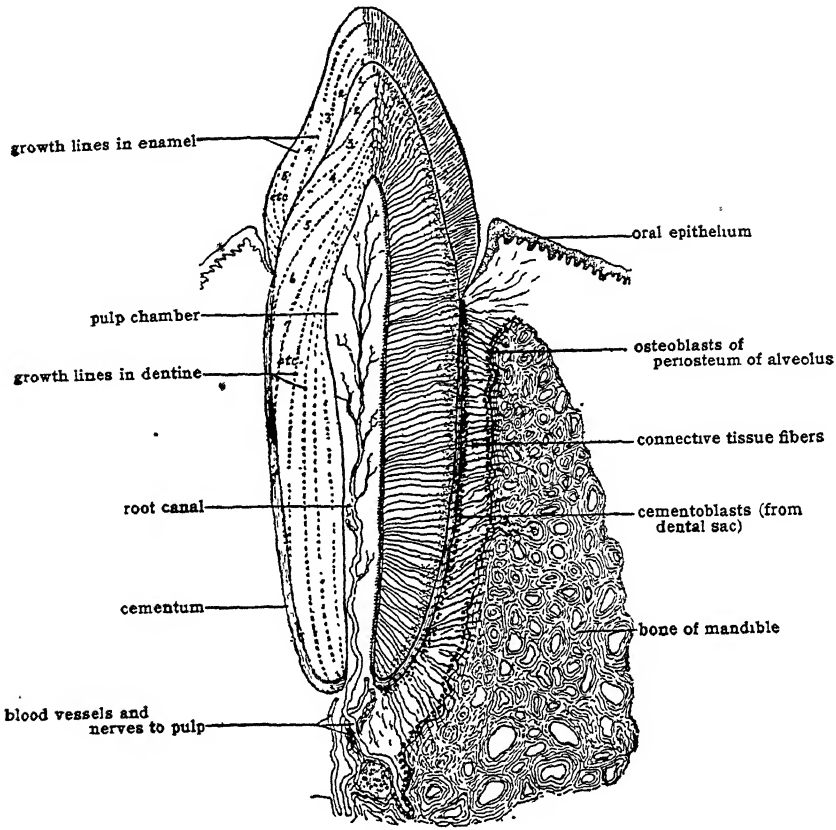


Fig. 107.—Vertical section of a tooth. (*Patten.*) (Bundy's Anatomy and Physiology, revised by Weeder)

Analyses of whole, sound teeth (human) give the following figures (Brekhus and Armstrong) (Table 77):

TABLE 77.—RANGE OF CONSTITUENTS IN PERCENTAGES ON "DRY" AND FAT-FREE BASIS.

Calcium	26.05–30.07
Magnesium	0.31– 0.89
Phosphorus	12.96–19.71
Carbon dioxide (from carbonate)	2.14– 3.13

The view held at one time that enamel is a "lifeless, inert, mostly inorganic substance" has undergone modification. This has become possible because of the application of radioactive isotopes for the study of mineral metabolism. Hevesy showed that a certain amount of the administered radioactive phosphorus did enter the enamel, though in not nearly so large a quantity as its penetration of dentin.

Metabolism.—A contribution to the metabolism of teeth was made by Hevesy who showed that when compounds of radioactive phos-

phorus were injected into animals, radioactive phosphorus was found in the whole teeth. The enamel contained about 10 per cent of the radioactive element present in dentin.

For the teeth to calcify properly the diet must include enough calcium and phosphorus and some of the vitamins (A, C, D). Some of the hormones are also important.

Fluorine and Dental Caries (see p. 429).—Armstrong has shown that there is more fluorine in the enamel of sound teeth than in the enamel of carious teeth. This suggests that an optimum intake of fluorine is related to dental caries.*

Such a view has been strengthened by a field study which concluded that there is a lower incidence of caries in regions where the fluorine content of the water is relatively high or in areas of endemic mottled enamel (p. 430). Further, the teeth of rats develop fewer caries when fluorine is added to the diet, either during the time of development of the teeth or after the teeth are mature.

References

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For a discussion of the association of adenosinetriphosphatase with myosin, see Green and Colowick, *Ann. Rev. Biochem.*, **13,** 158 (1944); Bailey, *Advances in Protein Chemistry*, **1,** 297 (1944).

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Epithelial tissue. Block and Horwitt, *J. Biol. Chem.*, **121,** 99 (1937) (the basic amino acids of keratins); Danforth, *Physiol. Rev.*, **19,** 94 (1939) (physiology of human hair); Block, *J. Biol. Chem.*, **128,** 181 (1939) (composition of keratin); Clay, Cook and Routh, *J. Am. Chem. Soc*, **62,** 2709 (1940) (composition of human hair); Routh, *J. Biol. Chem.*, **135,** 175 (1940) (chemical studies of wool); Edwards and Duntley, *Am. J. Anatomy*, **65,** 1 (1939) (pigments of the skin); Meyer and Chaffee, *J. Biol. Chem.*, **138,** 491 (1941) (mucopolysaccharides of the skin).

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A red pigment in human red hair is reported by Rothman and Flesch, *Proc. Soc. Exp. Biol. Med.*, **53,** 134 (1943).

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Bone. Huggins, *Physiol. Rev.*, **17,** 119 (1937) (composition of bone and function of the bone cell); Logan, *Physiol. Rev.*, **20,** 522 (1940) (chemistry of calcifica-

* The mean fluorine content of enamel and dentin of sound teeth is, respectively, 0.0111 and 0.0169 per cent. The fluorine content of mottled teeth is much higher; for example, enamel, 0.0245 to 0.0361, and dentin, 0.0371 to 0.0406.

tion); Hevesy, Levi and Rebbe, *Biochem. J.*, **34,** 532 (1940) (radioactive P and bone metabolism).

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Armstrong, *Federation Proceedings* (Fed. Am. Soc. Exp. Biology), **3,** 201 (1944), is the author of an article on bone metabolism.

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Deakins and Burt, *J. Biol. Chem.*, **156,** 77 (1944) deal with the deposition of calcium, phosphorus and carbon dioxide in calcifying dental enamel.

Cox, *Vitamins and Hormones*, **2,** 285 (1944), deals with fluorine and dental caries.

CHAPTER 23

URINE

The Kidneys.—The kidney is the chief organ of the body for the elimination of water and a number of nonvolatile substances. In a very general way, it serves the function of maintaining the composition of the fluids of the body at a certain level. This function is shared with the respiratory system, the skin, and the gastro-intestinal tract. To

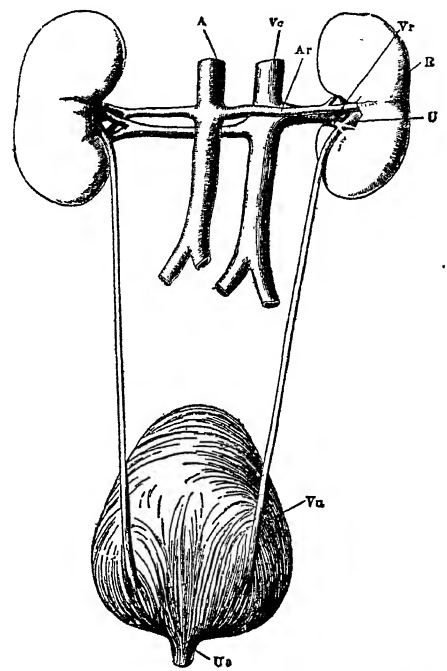


Fig. 108.—The urinary tract, viewed from behind. R, Right kidney; U, ureter; A, aorta; Vc, inferior vena cava; Ar, right renal artery; Vr, right renal vein; Vu, urinary bladder; Ua, first portion of uretha. (Henle.)

maintain a fluid of constant composition within the body, the kidney eliminates urine which, from time to time, varies very much as to composition, and which also varies as to rate of production. A constituent such as urea is found in far higher concentration in urine than in blood; and, under normal conditions, not more than a trace of glucose is found in the urine, although there are appreciable quantities in the blood.

The kidneys represent a complex gland made up of innumerable small tubes, the uriniferous tubules. From each kidney a tube, the

ureter, carries urine to the bladder (Figs. 108 and 109); and by means of another tube, the urethra, the urine is voided.

At the beginning of the uriniferous tubule is a capsule, known as Bowman's capsule, which surrounds a tuft of capillaries, the glomerulus. What is known as a "malpighian corpuscle" is made up of such a glomerulus and a Bowman's capsule.

To each capsule is attached a long tubule, including a convoluted tubule terminating in the "loop of Henle," and an ascending loop

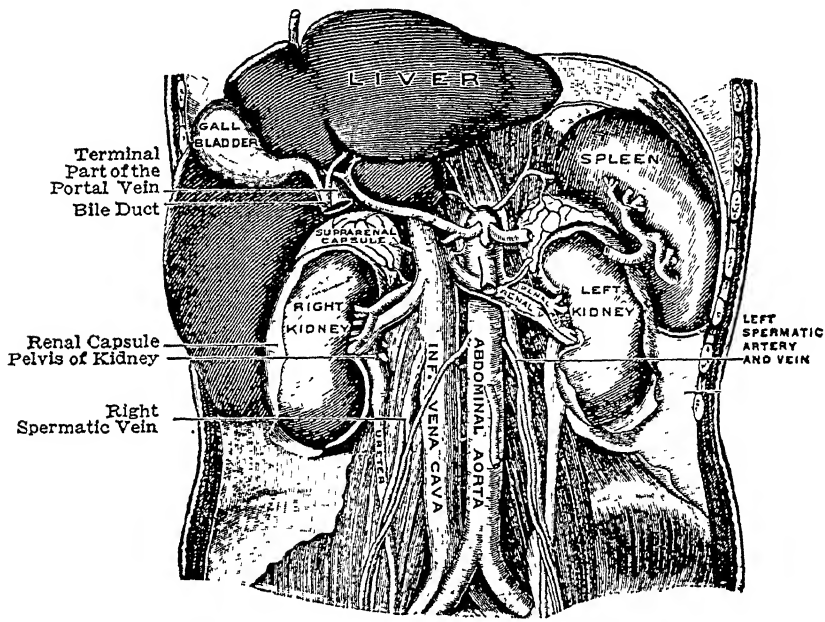


Fig. 109.—Organs of the abdomen; the stomach and intestines have been removed and the liver raised. (After Sappey.)

which ultimately connects with a main collecting tube opening into the renal pelvis (Fig. 110).

The Formation of Urine.—There have been many theories to explain the process by which urine is formed. It is generally agreed that at least two processes are involved: one includes a filtration process through Bowman's capsule, and the other, a process of concentration as the liquid passes the tubules, until what is known as "urine" is formed. Richards emphasizes these facts as follows: the kidney, first of all, has to separate a filtrate from the blood so enormous in volume as to contain the waste products of metabolism and the unneeded salts and water. But were the activities of the kidney to stop here, death would result from dehydration and loss of bases. This is prevented by a process of reabsorption through the different segments of the tubules. In this way, the water, salts, and diffusible nutrients needed by the body are absorbed again. Chemical (hormonal?) messages inform the tubule cells of changes in the composition of the blood. A mechanism promptly comes into play to prevent an abnormal composition.

Functions of the Kidney.—The important function of helping to maintain a uniform composition of the blood has already been alluded to. This work involves a number of activities: the excretion of substances which may be present in excessive quantities (for example, water, sugar); the excretion of substances no longer useful to the body (such as urea and uric acid); the excretion of salts to maintain the proper osmotic conditions in the blood; the excretion of acid or bases

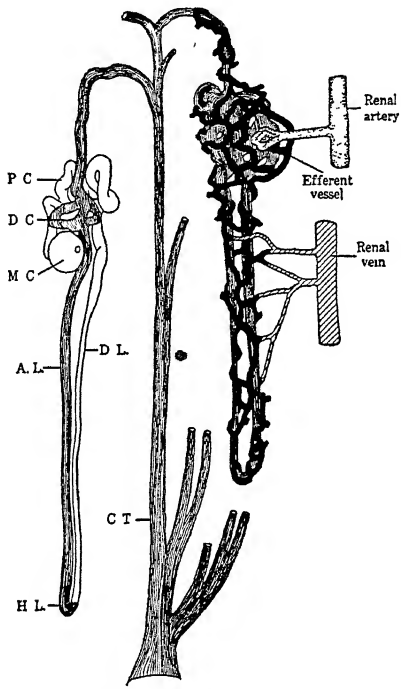


Fig 110 —Diagram of the tubular structure of the kidney. On the unit on the right the blood circulation is indicated. *M. C*, Malpighian corpuscle, *P. C.*, proximal convoluted tubule; *D L*, descending limb of Henle's loop, *H. L.*, Henle's loop; *A. L.*, ascending limb of Henle's loop; *D C*, distal convoluted tubule; *C. T.*, collecting tubule. (Cushny, *Secretion of the Urine*, Longmans, Greens and Co., Publishers)

to maintain the acid-base balance of the body; the detoxication and subsequent excretion of toxic substances (for example, the conversion of benzoic acid into hippuric acid, and the subsequent elimination of the latter).*

Renin, a Substance in the Kidney Which Affects Blood Pressure.—One of the commonest diseases suffered by man is arterial hypertension. While much remains to be learned as to the cause of this ailment, the work of Goldblatt suggests one more function of the kidney.

Goldblatt discovered that persistent hypertension could be induced in the dog by constriction of both main renal arteries; or by the constriction of one renal artery and the excision of the opposite kidney. That some active substance was discharged by the kidney into the

* This also occurs in the liver.

blood was made probable by several experiments. One of these consisted in transplanting a kidney to the neck, with no nervous connections with the rest of the body; a rise of blood pressure still occurred when the main artery to the kidney was constricted. This indicates that some active substance is released by the kidney into the systemic circulation.*

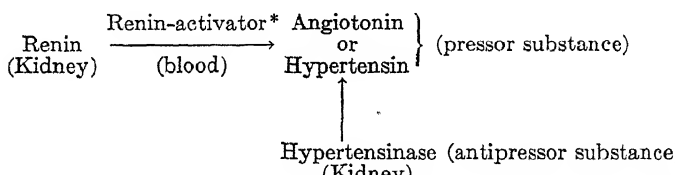
The substance responsible for this rise in blood pressure has been given the name *renin*. Various attempts at purification suggest that this *renin* is a protein, probably a globulin.

While epinephrine (p. 497), pituitrin (p. 492) and tyramine (p. 232) all cause a rise in blood pressure, they differ from renin in that the response to an intravenous injection of the latter is not associated with a decrease in peripheral blood flow or fall of skin temperature.

Renin is not effective unless injected intravenously. If, for example, it is incorporated in Ringer's solution (p. 422) and so perfused through an isolated organ, it shows no vasoconstrictor properties. It is not directly a pressor substance.

If, however, the perfusion experiment is repeated, using blood plasma in the place of Ringer's solution, there is marked constriction of the blood vessels. There is a "something" in blood which activates the renin. Page calls this "something" a *renin-activator*. The interaction between renin-activator and renin gives rise to the pressor substance, to which the name *angiotonin* has been given. It has also been named *hypertensin*.†

To prevent confusion, let us summarize by means of a diagram:



* Also called renin-substrate; hypertensinogen; pre-angiotonin, pre-hypertensin; blood plasma globulin.

† Also called angiotonase.

That renin is intimately connected with a rise in blood pressure is further confirmed by the demonstration that extracts of kidneys of animals with chronic renal hypertension contain more renin than extracts of the normal kidneys.

Chemical work on the renin-activator suggests that it also is a protein, and also of the globulin variety.

While renin and renin-activator are nondialyzable and thermolabile, angiotonin is dialyzable and thermostable. It has been suggested that renin is an enzyme, that it acts on renin-activator, to produce angiotonin, which is chemically a degradation product of a protein.

* "Although the kidney is not generally looked upon as an endocrine organ, there is no reason to deny the possibility of its performing such a function; for the elaboration of humoral substances is not limited to specifically endocrine organs" (Grollman, Harrison and Williams, Jr.).

† Experiments by Croxatto indicate that pepsin at *p*H 2.0 reacts with Page's "renin-activator" to form a substance similar to, if not identical with, angiotonin.

Using synthetic substrates of the type which Bergmann has employed so successfully in his study of proteolytic enzymes (p. 92), Page finds that his renin—probably still quite crude—contains several proteolytic enzymes such as pepsin, trypsin, carboxypeptidase and aminopeptidase.

The next advance in this field offers therapeutic possibilities. It can be shown that normal blood serum destroys angiotonin (or hypertensin) *in vitro*. This has been attributed to the presence of an enzyme, to which the name *hypertensinase* has been given.

That the kidney is the source of hypertensinase in normal blood is made probable by the fact that the substance disappears almost com-

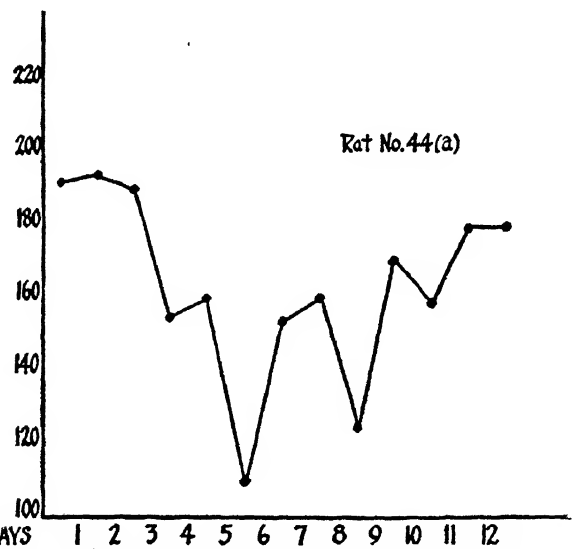


Fig. 111.—Effect of kidney extract subcutaneously administered on the arterial pressure of a hypertensive rat. (Page, *J. Urology*, 46, 823.)

pletely from the blood stream of dogs whose kidneys have been removed (bilaterally nephrectomized dogs).

Under normal conditions, according to one theory, there is a fine balance of forces between renin and the production of angiotonin, with a tendency to increase blood pressure, and hypertensinase, which, by destroying angiotonin, tends to lower blood pressure. Under normal conditions, then, any tendency in the one direction is counterbalanced by a tendency in the opposite direction; or a tendency to neutralize the effect. Under abnormal conditions, in hypertension, we may suppose that the discharge of renin and the production of angiotonin is not counterbalanced by a sufficient amount of hypertensinase. The deficiency here lies in this latter substance. " . . . Renal hypertension may be dependent on a disturbed balance between pressor and antipressor substances formed in the kidney" (Grollman, Harrison and Williams, Jr.).

Page, Grollman, and others have succeeded in preparing renal ex-

tracts (containing hypertensinase?) which were found active in lowering blood pressure.*

The extracts were first tried on hypertensive dogs and rats (Fig. 111) and then on hypertensive patients. The results were encouraging. "Treatment of patients with extract," writes Page, "must still be considered an experimental measure. . . . The patients appear to be greatly benefited, but it would be rash to assume after a study of only two years that these benefits will persist."

Schroeder has attacked the problem from a slightly different angle. He finds that tyrosinase (p. 399) destroys angiotonin *in vitro;* and since tyrosinase is a phenolic oxidase, it is postulated that angiotonin —like epinephrine (p. 497) and tyramine (p. 232)—contains a phenolic group.

Cruz-Coke claims that oxidized cytochrome destroys angiotonin—or hypertensin as he calls it—whereas reducing agents, such as ascorbic acid and cysteine, tend to retard destruction.

The claim has also been made by Grollman that fish oils contain the substance which reduces blood pressure in hypertensive rats. The effective substance is not vitamin A.

"Arteriosclerosis is the primary disease," writes Scott. "This process, when affecting the vascular system of the kidneys, excites a humoral mechanism which produces a widespread vasoconstriction and thus causes hypertension. Whether this is the benign or malignant type depends on the progress and severity of the vascular lesions in the kidneys. Essential hypertension is a manifestation of renal vascular disease, and therefore it appears as one aspect of human arteriosclerosis, the nature of which is, as we all know, one of the greatest unsolved problems in medicine today."

Composition of Urine.—A diagram representing comparative concentrations of substances in blood plasma and urine is instructive (Fig. 112):

The kidney is the main organ of regulation of extracellular fluid, which, as may be remembered (p. 272), consists of plasma and interstitial fluid including lymph. A substance like urea is in far higher concentration in urine than in blood. Substances like protein and glucose are not found in normal urine to any appreciable extent. Ammonia (in the form of ammonium salt) is present in urine and probably absent in blood.

Using very rough figures, and always working with a twenty-four-hour sample of urine, we may say that the average amount of urine voided during this period would be about 1500 cc. In this 1500 cc. of fluid would be found some 60 gm. of solids. Roughly, one half (or 30 gm.) is due to urea, and one quarter (or 15 gm.) is due to sodium chloride. The remaining 15 gm. includes the various organic and in-

* The outline of the method by Page is to use acetic-acid-saline extracts of ground kidneys, heat the extracts (to remove inactive proteins and lipids), fractionally precipitate with ammonium sulfate, extract with ether and treat with kaolin.

organic constituents (uric acid, creatinine, hormones, enzymes, vitamins, ammonia, sulfates, phosphates, etc.).

Under pathological conditions, substances appear in the urine which are normally absent, or, if anything, present in "traces"; these include proteins, sugar, acetone bodies, bile, hemoglobin, etc.

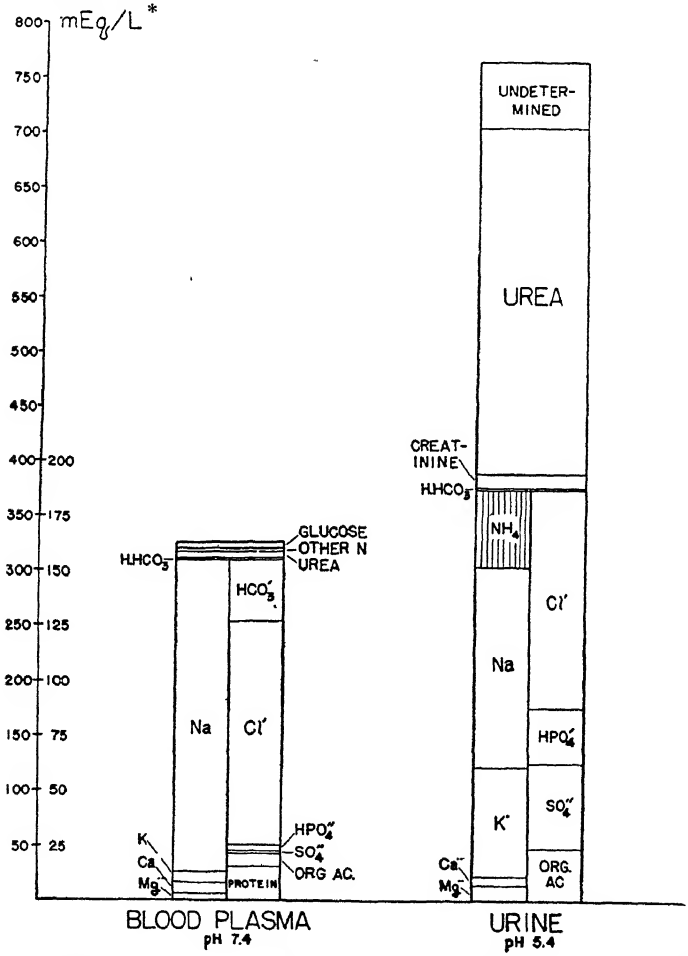


Fig. 112.—Comparison of composition of blood plasma and urine. [Gamble, *Extracellular Fluid* (Harvard Medical School, 1942).]

Some General Considerations.—The organic substances present undergo decomposition rapidly when the urine is left standing. For example, urea is changed to ammonia. It is important, therefore, in examining urine with a view to a quantitative analysis of its constituents, to work with "fresh" urine; or, since that is usually difficult, to use a urine to which a preservative has been added. Such preservatives include boric acid, formalin, thymol, toluene, chloroform,

*mEq/L = milliequivalents per liter. For sodium ion, for example, milliequivalents per liter is obtained by multiplying the mg. per 100 cc by 10 and dividing by 23.

etc. All of them are objectionable. The objection is usually due to some interference with a chemical test. Thymol and toluene are probably the most widely used.

Quantity.—As has already been stated, the quantity of urine voided in twenty-four hours may be some 1500 cc. Of course, this figure varies considerably with different individuals. The fluctuations for a normal adult are probably from 1200 to 1500 cc. (40–50 ounces). Increases beyond the normal amount (polyuria) occur in a number of diseases—chronic nephritis, diabetes, etc. In diabetes, the amount of urine voided in twenty-four hours is from 2 to 5 liters, and, in very rare cases, it may even reach 20 liters. The reversed condition, the elimination of a decreased quantity of urine (oliguria), occurs in diarrhea, fevers, etc.

Color.—The color of the urine, usually from yellow to reddish-yellow, will vary with the amount of urine voided. The chief pigment is urochrome (yellow in color). Small quantities of urobilin and hematoporphyrin are also present. With the presence of abnormal constituents, the color may change considerably. The presence of hemoglobin will give rise to a brown to red color. Bile in the urine may produce a yellow foam when the sample is shaken, and the color of the urine may become a pronounced brown. Rhubarb, cascara, and some other cathartics produce a brown color, which changes to red upon the addition of alkali.

"Fresh" urine is transparent. After a time, a cloud appears, due to the separation of mucus, leukocytes, and epithelial cells. Where there is much cloudiness, the effect may be due to phosphates, urates, pus, blood or bacteria. It must be emphasized at this point that normal urine, on standing, becomes alkaline; and this itself causes a precipitation of phosphates.

Odor.—The peculiar odor of urine is ascribed, rather vaguely, to "volatile acids." Urine undergoing decomposition has an ammoniacal odor. Certain dietary ingredients and a number of drugs influence the odor. For example, the eating of asparagus will give rise to a urine with a particularly offensive odor.

Reaction.—The reaction of normal urine is usually on the acid side (about *p*H 6); but the variations are considerable, even in normal samples. An excess of protein in the diet, producing increased quantities of sulfate and phosphate, will tend to increase the acidity of the urine. The acidity is also increased in acidosis and in fevers (with a concentrated urine).

As has already been stated, the urine become alkaline on standing, due to the gradual conversion of the urea into ammonia. Where the freshly voided urine is alkaline, the reason must be sought elsewhere. It may be due to decomposition in the bladder—a decomposition of urea after the urine is secreted. It may also be the result of frequent vomiting. It may, on the other hand, signify nothing more than a temporary "alkaline tide," due to a full meal, or it may be the result of eating excessive quantities of fruit. In the latter case, the salts of organic acids give rise to an alkaline ash when oxidized in the body.

Three to 5 gm. of sodium bicarbonate is enough, in a normal individual, to produce an alkaline urine.

Specific Gravity.—The usual range is from 1.017 to 1.020. It varies, in general, inversely with the quantity of urine voided.

The specific gravity is low in chronic nephritis and in diabetes insipidus, and it is high in fevers and in diabetes mellitus.

NORMAL CONSTITUENTS

The variations within normal limits are given in Table 78.

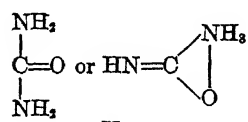
TABLE 78.—COMPOSITION OF NORMAL URINE.

	Gm. in 24 hours.
Water	1000–1500
Total solids	55–70
Urea	25–35
Creatinine	1.2–1.7
Uric acid	0.6–1
Amino acids	0 2–0.4
Phenols	0.1–0 2
Allantoin	0.005–0.015
Oxalic acid	0.01–0 02
Indican	0.005–0.01
Purine bases	0.01–0.06
Chloride (NaCl)	10–15
Sodium	3–5
Potassium	1.5–2.5
Calcium	0.1–0.3
Magnesium	0.1–0.2
Iron	0.001–0.005
Total sulfur (SO_3)	2.0–3.4
Inorganic sulfate (SO_3)	1.7–2.7
Ethereal sulfate (SO_3)	0.15–0.3
Neutral sulfur (SO_3)	0.2–0.4
Phosphate (P_2O_5)	2.5–3.5
Ammonia	0.5–1.0

(Also small quantities of volatile fatty acids, hippuric acid, purine bases, fluorides, nitrates, pigments, enzymes, vitamins, hormones, etc.)

A brief description of a number of these constituents will now be given.

Urea.—This substance represents the principal nitrogenous end-product. This is not true of all animals, as Baldwin has once again emphasized (Table 79).



Urea.

TABLE 79.—NITROGEN END-PRODUCT OF VARIOUS ANIMALS. (Baldwin, *Comparative Biochemistry*, Cambridge Univ. Press, London.)

Animal.	*Nitrogen end-product.*
Sharks, dog-fishes	Urea
Bony fishes (teleostei)	Ammonia
Frogs, newts	Urea
Turtles	Urea
Snakes, lizards	Uric acid
Birds	Uric acid
Mammals	Urea

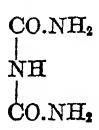
However, in man, for example, the output of urea varies directly with the protein intake; and usually constitutes from 80 to 90 per cent of the total nitrogen excretion. On a low protein diet, this ratio (80–90 per cent) is lowered (Table 80).

TABLE 80.—SHOWING THE AVERAGE AMOUNT OF DIFFERENT FORMS OF NITROGEN EXCRETED WHEN LOW, NORMAL AND HIGH PROTEIN DIETS WERE INGESTED. (Beard)

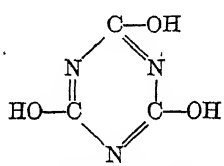
	Normal diet			High protein diet.			Low protein diet.		
Volume of urine Specific gravity	1364 cc 1 022			1472 cc 1 023			1408 cc. 1 019		
Substance.	Amount.	Nitrogen content.	Per cent of total N	Amount.	Nitrogen content.	Per cent of total N.	Amount.	Nitrogen content.	Per cent of total N
	gm	*gm*		*gm.*	*gm*		*gm*	*gm*	
Total nitrogen		11 16			15 28			7 97	
Urea	20 38	9 51	85.21	28 29	13 20	86 36	13 20	6 16	77.29
Ammonia	0 66	0 54	4 83	0 83	0 68	4 45	0 52	0 43	5 39
Uric acid	0 57	0 19	1.70	0 63	0 21	1 37	0 54	0 18	2 26
Creatinine	1.70	0 63	5.64	1 78	0 66	4 32	1 67	0 62	7 78
Undetermined nitrogen by difference		0 29	2.62		0 53	3 50		0 58	7 28

The formation of urea in the body has already been discussed (p. 357).

Urea is soluble in water and alcohol and insoluble in ether and chloroform. It forms biuret (Chap. 4) and cyanuric acid when heated.



Biuret.



Cyanuric acid.

It is oxidized by hypobromite in alkaline solution:

$$CO(NH_2)_2 + 3NaOBr \rightarrow 3NaBr + N_2 + CO_2 + 2H_2O$$

which forms the basis for a rough quantitative estimation of urea by measuring the volume of nitrogen eliminated. A much more accurate method for estimating this substance is based on the action of the enzyme *urease* (found in soy and jack beans), which quantitatively converts urea into ammonia.

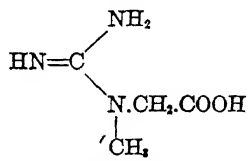
Characteristic crystals of urea nitrate, $CO(NH_2)_2.HNO_3$, and urea oxalate, $CO(NH_2)_2.H_2C_2O_4$, are easily obtained by mixing urea with the respective acids; these salts are of value for purposes of identification.

The amount of urea excreted is increased in fevers, diabetes (with little acidosis), etc. In diseases of the liver (acute yellow atrophy, cirrhosis, etc.), with a decreased formation of urea, there is less excreted. This is also true in cases of acidosis, where some of the nitrogen which would be normally converted into urea is changed to ammonia.

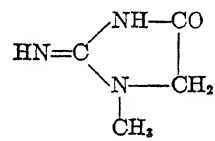
Aside from a decreased formation of urea with a subsequent decreased output, a retention of urea (as in nephritis) also leads to a smaller output. Here, for diagnostic purposes, the estimation of urea in the blood is of great importance.

Creatinine.—This substance—and its possible relationship to creatine—has already been discussed (p. 376). Creatinine is a normal constituent of urine; it is, according to Folin, relatively independent of the amount of protein ingested. Its amount, however, is decreased in many pathological conditions.

Creatinine is soluble in water and alcohol and forms a characteristic double salt with zinc chloride, $(C_4H_7N_3O)_2.ZnCl_2$, which is used for



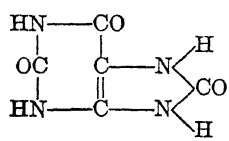
Creatine.



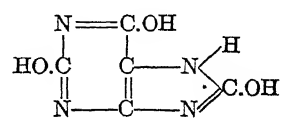
Creatinine.

the purpose of isolating the base. With picric acid, in an alkaline solution, it forms a red color; which is the basis for a colorimetric determination (Jaffe; Folin). Greenwald is of the opinion that the reaction is due to the formation of a red tautomer of creatinine picrate. In addition to this test, originally due to Jaffe, there is the test in which the urine (containing creatinine) is mixed with alkali and sodium nitroprusside, yielding a red color which turns yellow (Weyl); and if acetic acid is now added to the yellow solution and heated, a green, and finally a blue color (Prussian blue) is obtained (Salkowski).

Uric Acid (See p. 85).—This substance represents one of the final stages in the oxidation of the purines in the body and is the chief



Keto (lactam) form.



Enol (lactim) form.

Uric acid.

nitrogenous end-product in birds, snakes, and lizards (Table 79, p. 465). It is derived from the nucleoproteins of the food and from the breakdown of nucleoprotein within the cells of the body.

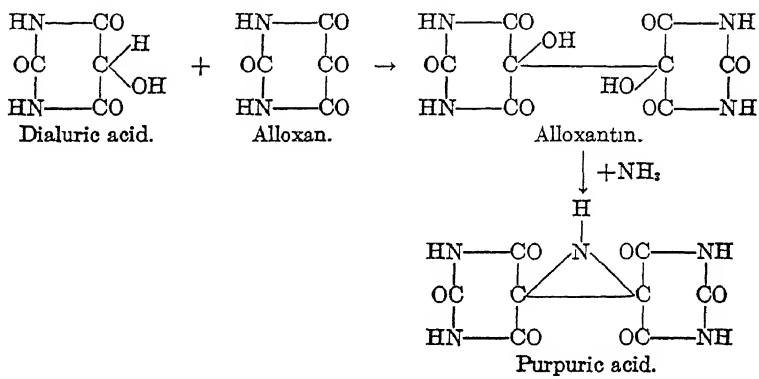
Uric acid acts as an acid (examine the "enol" formula) and forms salts with sodium and potassium to give the corresponding urates. It is these urates, very largely, which are found in the urine, the highly insoluble free acid being obtained on strong acidification. The

acid itself, however, is probably also present to some degree. The urates—the acid salts particularly—are thrown out of solution when urine is concentrated, giving the sediment of "amorphous urates."

Uric acid is but slightly soluble in water and insoluble in alcohol and ether. It forms soluble salts with alkalis.

In leukemia, with destruction of leukocytes, the uric acid output is very much increased. This is also true in diseases associated with the liver, an organ rich in nuclein material. The popular mind has long associated gout with a disturbed metabolism of uric acid; but the connection is not altogether clear. Before an attack, the output is somewhat decreased; and for several days after the attack, the output is definitely increased.

A characteristic test for uric acid is the *murexide reaction*. This is obtained by evaporating the uric acid with nitric acid and treating the residue with ammonia; a reddish-violet product (murexide, or ammonium purpurate) is obtained. The nitric acid oxidizes uric acid to dialuric acid and alloxan, which then condense:



Uric acid also reduces silver solutions in an alkaline medium (Schiff test) and gives a blue color with phosphotungstic acid (Folin), which serves as the basis for one quantitative procedure.

Amino Acids.—The small quantity normally in urine is much increased in impairment of hepatic function (as in yellow atrophy of the liver), eclampsia and in certain types of poisoning (such as that due to chloroform, phosphorus, arsenic or carbon tetrachloride).

Chlorides.—Next to urea, chlorides are the most abundant substances. The chlorides, mainly as sodium chloride, are derived chiefly from the food, and the output, therefore, fluctuates depending upon the intake. During starvation the output may be almost abolished, and yet the chlorides in the blood will maintain for a time their normal concentration.

There is a decrease in the elimination of chlorides in several forms of nephritis and in fevers.

Chlorides may be detected by the white precipitate of silver chloride which is formed when the solution and silver nitrate are mixed in the presence of nitric acid. A quantitative method for chlorides makes

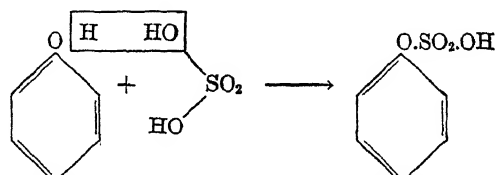
use of this reaction. Here an excess of silver nitrate is added, the silver chloride is filtered off, and the excess silver nitrate is titrated with a standard thiocyanate solution.

Sulfates.—Most of the sulfur has its origin in protein. Much of it is derived from the protein in the food, and some of it has its source in cellular activity.

The sulfur appears in the urine in three forms: inorganic sulfate; ethereal sulfate; and "neutral" sulfur.

Inorganic Sulfates.—Roughly speaking, the output of sulfate is proportional to the output of total nitrogen, the ratio N/SO_3 is about 5/1. Since the amount of nitrogen eliminated (as urea, etc.) is a measure of the amount of protein metabolized, estimations of inorganic sulfate as well as of nitrogen are valuable in studies dealing with protein metabolism.

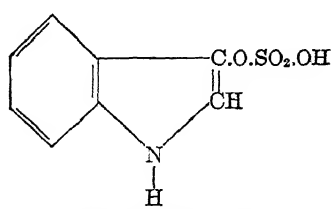
Ethereal Sulfates.—Of the total, preformed sulfates present in urine, about nine-tenths is in the inorganic form (combined with Na, K, Ca, and Mg). About one-tenth, however, is in the form of an ester: a combination of sulfuric acid with phenols:



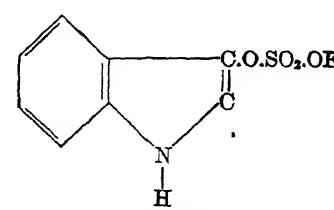
Phenol. Phenolsulfuric acid.

Other substances combined with the acid are *p*-cresol, indole (as indoxyl) and skatole (as skatoxyl). All these are included under the name "ethereal sulfates."

The ethereal sulfates represent, to some extent, the putrefactive products in the intestine which are detoxified in the liver and then eliminated. To a large extent, these substances are the result of normal protein metabolism in the body; but the exception, according to Folin, is indican, which really represents putrefactive activity. Indican is the potassium salt of indoxyl sulfuric acid:



Indoxyl sulfuric acid.

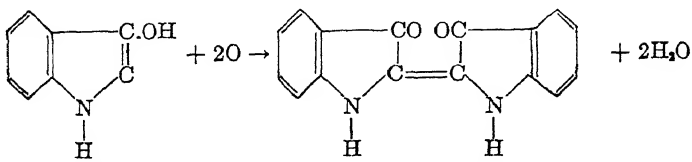


Indican.

Indicanuria (a substantial increase in the output of indican) is common in diseases of the small intestine (intestinal obstruction, for example), and in intestinal indigestion ("biliousness"). Simple constipation of the large intestine is not, as a rule, followed by an indicanuria. Increases in indican are also noted in diseases of the stomach in

which there is a subnormal amount of hydrochloric acid (gastritis, cancer).

The tests used in detecting indican in the urine depend upon its decomposition and oxidation to indigo-blue (Jaffe; Obermayer):



Indoxyl. Indigo-blue.

In the Obermayer method, the urine is mixed with the reagent (ferric chloride and concentrated hydrochloric acid), chloroform is added, and the mixture is shaken. The chloroform turns blue, the intensity depending upon the amount of indican present.

Acidification of urine with hydrochloric acid and the addition of barium chloride precipitates inorganic sulfates. This precipitate is filtered off, and the filtrate is heated. If an excess of barium chloride has been added, a second precipitate will be formed. The hot acid hydrolyzes the ethereal sulfates, and the sulfate ion combines with the barium ion. This method is used not only qualitatively for the detection of the two types of sulfur, but is the basis for a quantitative determination.

"*Neutral Sulfur.*—This represents sulfur in an incomplete state of oxidation, possibly in the form of cystine, taurine, sulfides, thiocyanates, etc. Its amount seems to be independent of the amount of protein ingested; and in this sense it resembles creatinine.

This type of sulfur can be tested for by adding zinc and hydrochloric acid to a sample of urine. The hydrogen combines with the sulfur, and the hydrogen sulfide which is liberated is identified with a strip of paper soaked in a solution of lead acetate. Black lead sulfide is formed.

The quantitative determination of "neutral" sulfur is carried out by evaporating a sample of urine to dryness, heating the residue with an oxidizing mixture (a mixture of copper nitrate and potassium chlorate, or just sodium peroxide alone), thereby converting all the sulfur to sulfate. By precipitating with barium chloride, the total sulfate can be determined. If we now subtract the inorganic and etheral sulfates from the total sulfate, the difference represents the "neutral" sulfur.

Phosphates.—These substances are very largely derived from the foods we eat, though a small quantity has its origin in cellular metabolism. There are two types of phosphates, the alkaline and the earthy phosphates. The alkaline phosphates, which make up some two-thirds of the whole, are salts of sodium and potassium; and the earthy phosphates are combinations of calcium and magnesium. In alkaline urines, the "amorphous" phosphate precipitates are due to the alkaline earth variety. The ammonia formed when urine is exposed for a time com-

bines with the magnesium and the phosphate to form ammonium magnesium phosphate, or "triple phosphate," which is an insoluble and characteristically crystalline product.

It is claimed that the acidity of fresh, normal urine is partly, though not wholly, due to the presence of NaH_2PO_4. In urine the acid salt predominates, whereas in blood the basic salt, Na_2HPO_4, is present in large quantity.

From the clinical point of view variations in the phosphate content of urine are not very important; here studies in blood chemistry yield results of greater significance. But it should be mentioned that in bone diseases (rickets, osteomalacia) there is an increased excretion of phosphorus; and sometimes a decreased output has been noted in infectious diseases and in diseases of the kidneys.

The two types of phosphates can be detected by first precipitating the earthy phosphates by the addition of ammonium hydroxide, filtering, adding magnesia mixture (magnesium sulfate + ammonium chloride + ammonium hydroxide) to the filtrate, and warming; the white precipitate so obtained represents the alkaline phosphates.

Several methods are available for the determination of phosphate. One such method depends upon the addition of molybdate solution (sodium molybdate in sulfuric acid) to form phosphomolybdate, which is then reduced to a blue compound by means of stannous chloride; and the intensity of the blue color can be estimated colorimetrically.

Ammonia.—Ammonia is excreted as ammonium salts in amounts which tend to adjust the acid-base balance of the body. Unless ammonia (as ammonium ion) were available, acids might use too much of the fixed bases of the blood (represented by Na and K) and so endanger blood neutrality. This adjustment comes admirably into play when acids or foods yielding acids in the body are fed; the amount of ammonia excreted is increased. On the other hand, when alkali or foods yielding bases in the body are ingested, the excretion of ammonia is decreased.

The origin of this ammonia was explained in this way: normally amino acids are deaminated and most of the ammonia which results is converted into urea, a small portion escaping as ammonia. The danger of acidosis diverts some of the ammonia which normally would be used to form urea into combination with acid radicals, resulting in increased ammonia and decreased urea output.

This theory was attacked by Benedict who claimed that urinary ammonia had its source in urea, and that the latter was converted into ammonia in the kidneys. The kidneys, however, do not contain any urease, which made it difficult to understand how such a conversion could take place.

The discovery that the kidneys as well as the liver could deaminate amino acids suggested that the source of urinary ammonia could be traced to amino acids in general—a revival of the older view.

The recent work of Van Slyke and his associates may possibly settle the problem. Using explanted kidneys, they determined the amounts of materials removed from the blood per unit of time. All of

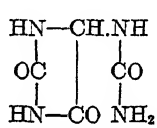
the urea in the blood found its way into the urine; there was apparently no conversion into ammonia. The small amount of α-amino acids removed from the blood failed to account for the amount of ammonia in the urine.

However, one amino acid in the form of its amide, glutamine (p. 372), is apparently the main source of urinary ammonia. The amount of glutamine in the blood was enough to account for all the ammonia in the urine (conversion of glutamine to glutamic acid plus ammonia).

When glutamine was administered to a dog suffering from an experimentally-induced acidosis (with HCl), the amount of ammonia excreted was increased. On the other hand, in a dog suffering from alkalosis—induced with sodium bicarbonate—the amount of glutamine removed from renal blood decreased.

One method of estimating the ammonia in the urine is to liberate it from its ammonium salt (by the addition of alkali), and to aerate the ammonia so liberated into an excess of standard acid. The acid left unneutralized is titrated with standard alkali.

Various Other Constituents.—*Allantoin*, a partial oxidation product of uric acid, is present in very small quantities in human urine; but in other mammals (except the anthropoid ape) it is the chief end-



Allantoin.

product of the metabolism of purines. It will be remembered that in the human being, the chief end-product of purine metabolism is uric acid.

The efficacy of the rather ancient treatment of healing wounds with maggots is now attributed to the formation of allantoin.

Oxalic acid, $(COOH)_2$, is found in the form of calcium oxalate. This rather insoluble salt is kept in solution by the presence of the acid phosphate. The source of the acid is believed to reside in the food we eat. Cabbage, grapes, lettuce, tomatoes, etc., contain oxalates.

Several purine bases, representing substances which have not been oxidized to uric acid, are found. Some of them are derived from the caffeine and theobromine found in coffee and tea.

For references to sodium, potassium, calcium, magnesium, and iron, see Chapter 21.

ABNORMAL CONSTITUENTS

Under pathological conditions, a number of substances are found in the urine which, normally, are hardly found at all. Among such substances are glucose, protein, acetone bodies, etc. Brief descriptions of some of these will now be given.

Proteins.—What is known as the "albumin" of the urine is really a mixture of serum albumin and serum globulin. An "albuminuria" is commonly attributed to damaged kidneys (nephrosis), such as an

inflamed organ (nephritis). As much as 20 gm. of protein may be eliminated in twenty-four hours.

The albumin may be detected by heating the urine, then adding a little dilute acetic acid; a white cloud (or precipitate) is formed. The several methods of estimating the protein depend upon a preliminary precipitation of the protein with trichloracetic acid or some other "alkaloidal" reagent. In one such method (Van Slyke), the protein is precipitated with trichloracetic acid, dissolved in sodium hydroxide, and copper sulfate added. The intensity of the color formed ("biuret") is estimated colorimetrically.

Bence-Jones Proteinuria, often associated with multiple myeloma (tumor-like hyperplasia of the bone marrow) is due to a peculiar protein in urine which precipitates at a low temperature (50 to 60° C.) and is dissolved—to a greater or less extent—at 100° C., the precipitate forming once again upon cooling. This protein is said to belong to the globulins.

Glucose (Dextrose).—Appreciable quantities of this sugar in the urine indicate a "glycosuria." What is known as "renal glycosuria" is due to a lowered renal threshold; which means that although sugar appears in the urine, there is no increase of sugar in the blood. An increase of sugar in the blood (hyperglycemia), with a corresponding elimination of sugar in the urine, is found in diabetes. In most instances, a glycosuria is indicative of diabetes. In this condition, the sugar will vary from 3 to 5 per cent, although sometimes it is even higher.

The Benedict test for glucose has already been discussed (Chap. 2). This is also the basis for a quantitative estimation.

Acetone Bodies.—These have been discussed in connection with fat metabolism (Chap. 17). While these substances are present in normal urine in "traces," in pathological conditions they may increase from 0.02 to 6 gm., of which β-hydroxybutyric acid often forms a large percentage. This last substance, together with acetoacetic acid, is eliminated as a salt, and so depletes the alkali reserve of the body; giving rise to an acidosis. To meet the crisis more ammonia is formed in the kidney.

The qualitative tests for "acetone bodies" are, as a rule, tests for acetone; and as acetoacetic acid very easily decomposes into acetone, the tests also include this acid. One such test is based on the transformation of acetone into iodoform: the urine is heated with sodium hydroxide and iodine; a precipitate of iodoform is formed.

If the urine is fresh, a test for acetoacetic acid may be shown by obtaining a reddish-colored solution with ferric chloride. The test for β-hydroxybutyric acid is seldom carried out, since it is somewhat involved, necessitating, first, the removal of the other two "acetone bodies."

One method of determining these substances quantitatively is to convert the two acids to acetone and precipitate the latter as a basic mercuric sulfate (Van Slyke). Given a mixture containing acetone (1), acetoacetic acid (2) and β-hydroxybutyric acid (3), the mere heating

of such a mixture will convert (2) into (1); and if, in addition to the heating, an oxidizing agent is present (such as dichromate), (3) is also converted to (1). This procedure, then, gives "total acetone bodies," If we wish to determine (1) and (2) alone, the dichromate is omitted. On the other hand, by making use of the relative volatility of (1) and (2)—they may be removed by heating the acidified urine—(3) itself may be determined.

Bile.—An obstruction in the bile duct, preventing the normal outflow of bile and forcing it back into the general circulation, gives rise to "jaundice" or "icterus." The yellowness of the skin is due to the bile pigments, which also appear in the urine. The pigments may be detected by the play of colors obtained on the addition of concentrated nitric acid (Gmelin), the various colored products representing stages of oxidation of bilirubin.

Blood.—Blood in the urine (hematuria) may result from a lesion in the kidney or the urinary tract. This is more common than "hemoglobinuria," in which hemoglobin without the red corpuscles is recognized. Where the destruction of red blood cells is very great (as in bad burns), the liver cannot change all of the hemoglobin into bile pigments, and some of the blood pigment appears in the urine. The tests for hemoglobin have already been given (Chap. 13).

Tests for Kidney Function.—The appearance of protein, blood, etc., in the urine suggests degenerative changes in the kidney. Various tests have been suggested—some 50 such tests have been listed—for determining how effectively the kidneys perform their function. Two such tests will be mentioned. One of them, the phenolsulfonephthalein test, is based upon the fact that this drug is eliminated by the kidneys only and can be recognized by the red color produced on the addition of alkali; which makes a colorimetric determination possible.

Under normal conditions, from 60 to 75 per cent of the dye is eliminated in the first two hours. Where there is marked renal insufficiency, the figure may be 20, and even less.

Another test for kidney function, and one of the most satisfactory tests, is what is known as the "urea clearance," in which a comparison is made between the concentration of urea in the blood and the rate of its excretion in the urine. Essentially, the relationship can be expressed as

$$\frac{u}{B}\sqrt{V}$$

where u = urine urea nitrogen, in mg. per 100 cc
B = blood urea nitrogen, in mg. per 100 cc.
V = cc. of urine excreted per minute (usually 1 cc.).

The "urea clearance" drops sharply in cases of renal insufficiency.

References

Reviews dealing with the physiology of the kidney may be found in the *Ann. Rev. Physiol.*, **6**, 265 (1944) (Braun-Menendez); **7**, 331 (1945) (Lamport).

For practical handbooks dealing with the analysis of urine, see Todd and Sanford, *Clinical Diagnosis by Laboratory Methods* (1943), Chap. 2; Simmons, *Laboratory Methods of the U. S. Army* (1935), p. 1, Hawk and Bergeim, *Practical Physiological Chemistry* (1937), Chaps. 27–32.

Theories dealing with the secretion of urine are reviewed by Cantarow and Trumper, *Clinical Biochemistry* (1945), p. 341; Richards, *Harvey Lectures*, Ser. 30 (1934–1935), p. 93, *Bull. N. Y. Acad Med*, Jan., 1938; *Proc. R. S* (London), Series B, **126,** 398 (1938). See, also, Shannon, *Physiol. Rev.*, **19,** 63 (1939).

Papers dealing with renin and hypertension are legion. Here are examples: Goldblatt, Kahn and Lewis, *J. Am. Med. Assoc.*, **119,** 1192 (1942) (critical evaluation of kidney extracts); Page, Ibid, **120,** 757 (1942) (a description by one of the active workers); Lamport, *Ann. Rev. Physiol*, **7,** 345 (1945) (review); Scott, *J. Am. Med Assoc*, **120,** 2 (1942) (clinical); Smith, Goldring and Chasis, *Bull N Y. Acad. Med.*, July, 1943, p 449 (critical); Page, *Ibid*, July, 1943, p. 461 (in defense); Remington, Cartland, Drill and Swingle, *Am. J Physiol*, **140,** 627 (1944) (purification of active material); Cruz-Coke, Reyes and Mardones, *Proc Soc Exp. Biol. Med*, **58,** 196 (1945) (destruction of hypertension by cytochrome); Grollman and Harrison, *Ibid*, **52,** 162 (1943) (curative effect of fish oils); Plentl and Page, *J. Biol. Chem*, **155,** 363 (1944) (the proteolytic character of renin); Plentl and Page, *Ibid*, **158,** 49 (1945) (purification of angiotonin); Alonso, Croxatto and Croxato, *Proc. Soc Exp Biol Med*, **52,** 61 (1943) (use of pepsin in the place of renin).

For the evidence that glutamine is the source of urinary ammonia, see Van Slyke, Phillips, Hamilton, Archibald, Futcher and Hiller, *J Biol Chem.*, **150,** 481 (1943), *J. Am Med. Assoc.*, **124,** 577 (1944); *Ibid*, **126,** 1089 (1944).

CHAPTER 24

HORMONES

Most of the glands of the body have ducts. The secretions which these glands manufacture are poured out through such ducts. Typical examples are the salivary and gastric glands. Another group of glands (the endocrine organs, Fig. 113) discharge their secretions directly into

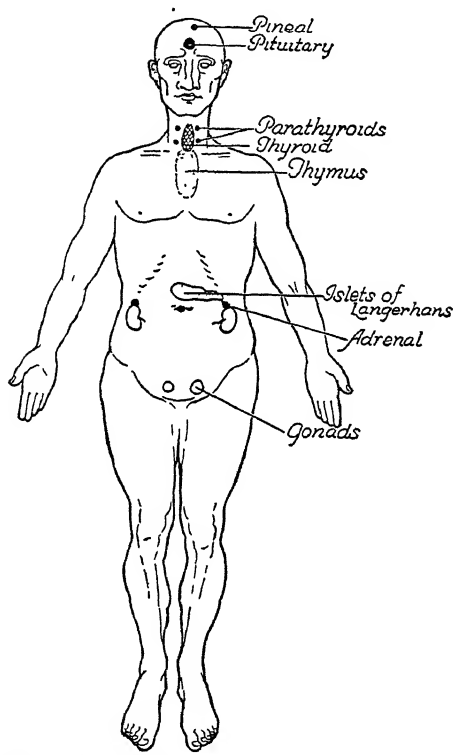


Fig. 113.—The location of some of the glands of internal secretion. (Williams.)

the blood; these secretions usually contain hormones. *Hormones* are definite chemical substances which are carried by the blood to various organs of the body to influence the activities of such organs. They are the "chemical messengers" of the body.

Some of the hormones are proteins (insulin, for example); others are related to the sterols (the male and female hormones); and still others are relatively simple substances (epinephrine and thyroxine).

We shall discuss these hormones in connection with the glands which manufacture them.

THE THYROID

The thyroid gland is made up of two lobes on either side of the trachea and the larynx. It weighs, on an average, from 16 to 23 gm. in the adult. Through its hormone it regulates, among other things, the rate of metabolism within the body. It also has other profound effects, as is seen in cases of "cretinism," where we have individuals who are mentally and physically retarded, and who suffer from a deficiency of the hormone (Figs. 114 and 115). Thyroxine, the hormone

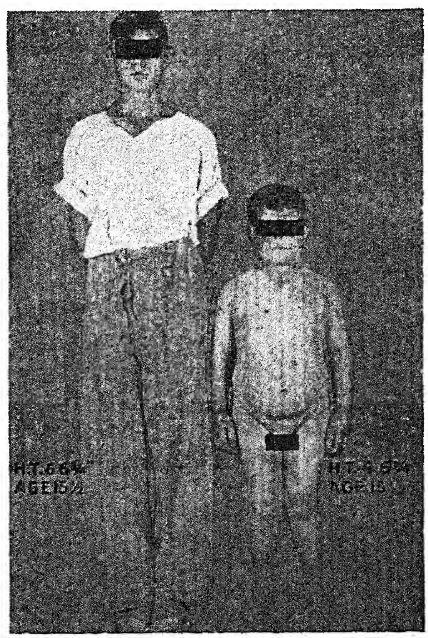


Fig. 114.—Comparison of cretin, aged fifteen and one-half years, with normal boy of the same age. (Werner, *Endocrinology, Clinical Application and Treatment*, Lea & Febiger, Publishers.)

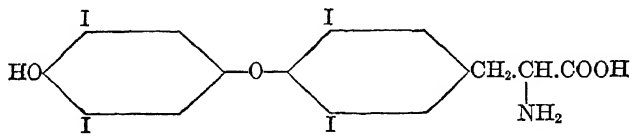
used, will cause remarkable cures. An enlargement of the gland, known as a goiter, may be of two kinds: "endemic goiter," due to insufficient iodine; or one due to an abnormally high activity of the thyroid gland, as in exophthalmic goiter and Graves' disease (Fig. 116). The "endemic goiter" lends itself to treatment with iodine (p. 429). Both hypo- and hyperactivities of the thyroid effect the basal metabolism (p. 411).

The treatment of endemic goiter—endemic because it occurs in places where the soil or water is deficient in iodine—with sodium iodide has been markedly successful. Marine and Kimball, pioneers in this field, discovered in the Akron-Cleveland district that about 45 per cent of the school girls from the fifth to twelfth grades and about 18 per cent of the boys showed goiter symptoms in various degrees. Treatment which involved the use of 2 gm. of sodium iodide, given in doses of 0.2 gm., and distributed over two weeks, caused complete cures.

Fig. 115.—Cretinism. A group of seven cretins at Urnatsch, Switzerland, showing their appearance and stunted growth compared to the normal individual in the center background. (Photographed by and reproduced through the courtesy of Professor J. F. McClendon. Grollman, *Essentials of Endocrinology*, J. B. Lippincott Co.)

Fig. 116.—Graves' disease. Marked exophthalmos in a patient suffering from severe hyperthyroidism and from whom a large diffuse goiter had been removed. (Dr. George Crile, Jr., *Surgery, Gynecology and Obstetrics*, **67**, 661.)

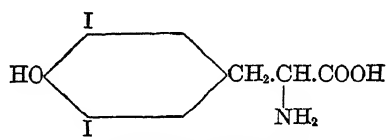
As early as 1895, Baumann made the discovery that the thyroid contains iodine—an element which until then was not suspected as one of the elements of the body. The active substance present in the thyroid gland is thyroxine, which was first isolated by Kendall and later by Harington. We owe to Harington its correct formula, as well as its synthesis.



Thyroxine.

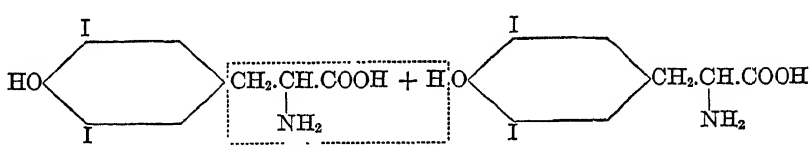
β-[3,5-diiodo-4-(3′,5′-diiodo-4′-hydroxy-phenoxy)-phenyl]-α-amino-propionic acid.

Origin of Thyroxine.—The thyroxine is not found as such in the thyroid gland but in the form of a protein, thyroglobulin. Harington is of the opinion that the gland manufactures this protein by first



3,5-Diiodotyrosine. *

forming 3,5-diiodotyrosine from iodine and tyrosine, which is then converted into thyroxine by the union of two molecules:



The thyroxine so formed,† together with unchanged diiodotyrosine, and other amino acids, finally forms a molecule of thyroglobulin.

That thyroxine is formed from diiodotyrosine has been made probable by the actual conversion of the latter into the former *in vitro*. Tyrosine was first converted into the diiodo salt, the latter dissolved in sodium hydroxide and maintained at a *p*H of 8.8 and a temperature of 70° C. for fourteen days. Thyroxine was precipitated with acid, purified and separated as the potassium salt.

Another striking conversion *in vitro*, but this time using thyroid tissue (from sheep, dogs and rats) was to show that radioactive iodine is taken up by diiodotyrosine and thyroxine. The thyroid tissue was sliced and added to a Ringer's solution (p. 422) containing 0.1γ of I^{127} per cc. (1γ = 0.001 mg). The organic iodine compounds were isolated and their radioactivity determined. In the rat some 12 per cent of

* Mono-(3-) iodotyrosine has also been recovered from iodinated casein.

† The alanine, which should also be formed, has not been isolated as such, but both pyruvic acid and ammonia—which, between them, would be the equivalent of alanine—have been obtained.

the radioactive iodine was incorporated into thyroxine and some 70 per cent into the diiodotyrosine.

That tissues other than the thyroid can synthesize the two organic compounds of iodine was shown by using completely thyroidectomized rats. These animals, devoid of all visible thyroid tissue, were injected with radioiodide, in due time anesthetized, tissues removed and the iodine compounds isolated. Ninety-six hours after the injection, 30 per cent of the radioiodine in the liver and small intestine were found to be in organic combination, some 20 per cent as diiodotyrosine and some 8 per cent as thyroxine.

But what tissues other than the thyroid can function in the formation of thyroxine? That is a question yet to be answered. Furthermore, it is somewhat disturbing to discover that the view that specific hormones are manufactured by specific tissues is not necessarily true. We have, for example, always associated the pancreas as the seat of the manufacture of insulin (p. 315) and the testicle as the seat of the manufacture of testosterone (p. 515). Are such views to be modified?

That oxidations of some kind are involved in the synthesis of thyroxine is supported by the evidence that substances which inhibit the activity of cytochrome and cytochrome oxidase (p. 390) also inhibit the formation of the hormone.

Thyroxine from Iodinated Proteins.—If casein (or some other protein) is "iodized"—if, in other words, it is treated in an alkaline solution with iodine at fairly high temperatures, the product shows definite thyroid activity. When this iodized protein is hydrolyzed and fractionated, diiodotyrosine and thyroxine can be separated.

Some details of this iodination of casein are of interest. In one such experiment, the casein was suspended in water containing 0.7 per cent sodium carbonate. The solution was heated to 40° C. and iodine, in small quantities, added over a period of several hours (17.6 gm. of iodine were added per 100 gm. of casein. This amount was calculated on the basis of the requirements for iodinating the tyrosine in the protein). The temperature was raised to 70° C. and held there for some 24 hours. By hydrolysis with barium hydroxide, thyroxine was eventually recovered from this mixture.

As might be anticipated, casein is not the only protein which, by iodination and subsequent treatment, yields thyroxine. Soybean protein serves equally well. Apparently the value of the protein for this purpose lies in its content of tyrosine.

Thyroxine and diiodotyrosine are the only two compounds of iodine known to exist in the thyroid. About 30 per cent is in the form of the hormone and about 70 per cent as the iodinated tyrosine.

Highly purified thyroglobulin, when analyzed, yields: cystine, 4.30 per cent; methionine, 1.31 per cent; tryptophan, 1.88 per cent; tyrosine, 3.00 per cent; diiodotyrosine, 0.67 per cent; thyroxine, 0.28 per cent; and glucosamine, 2.20 per cent.

It has been estimated that the average person contains the equivalent of about 14 mg. of thyroxine. The injection of 1 mg. of thyroxine will increase the basal metabolic rate by 2.8 per cent.

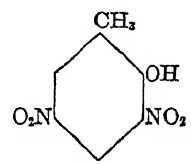
One of the hormones of the anterior pituitary, the *thyrotropic hormone* (p. 489), influences the activity of the thyroid gland. An injection of the thyrotropic hormone into a normal animal gives rise to hypertrophy and hyperactivity of the thyroid.

Treatment.—In cases of hypothyroidism, as in cretinism and myxedema, thyroxine or desiccated thyroid can be administered. These materials can be given orally, though of the two, the thyroid is the more easily absorbed and utilized. With the probable exception of one or two of the sex hormones, thyroxine is the only hormone which is so readily active when given orally; for insulin and other hormones have to be given parenterally.

Where there is hyperfunction of the thyroid, an increased elimination (in the urine) of iodine results. Sometimes the increase is from three to four times the normal amount. Estimations of iodine in blood and in urine may therefore be of value. The treatment is often one of surgery. Some four-fifths of the gland is removed, with the idea of lessening the production of thyroxine.

Another treatment still very much in its experimental stage is by the use of thiouracil (p. 482).

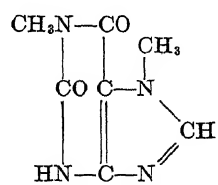
Nitrophenols.—Several nitrophenols—such as 4:6-dinitro-*o*-cresol—share the property with thyroxine of raising the basal metabolism but have no power of curing cretinism, or, what corresponds to it in the adult, myxedema. Since these dinitrophenols are toxic, their use in the attempt to reduce weight should not be encouraged.



4:6-Dinitro-*o*-cresol.

Antithyroid.—An important discovery by Carter, Mann, etc. strongly suggests that the basal metabolic rate is not exclusively controlled by thyroxine, but also by another substance, present in the body, the effect of which is in a direction opposite to that of thyroxine. This counterbalancing of forces is one of the marked characteristics of the activities within the body (see, for example, under renin, p. 459, and insulin, p. 493).

The antithyroid substance has been identified as paraxanthine, or 1,7-dimethylxanthine:

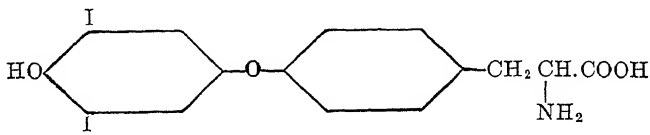


Paraxanthine.

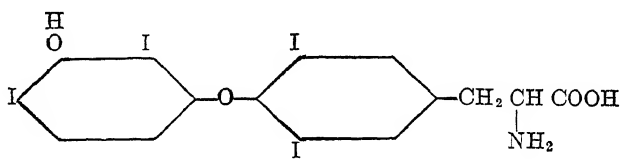
Vitamins and Thyroid Activity.—In hyperthyroidism the amounts of vitamins A, B and C needed are higher than in normal conditions.

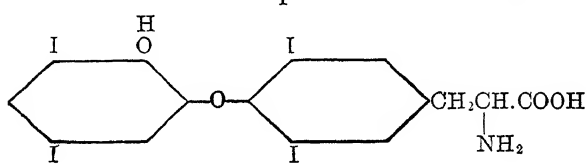
If such increased amounts fail to be given, a vitamin deficiency develops which has, at times, been confused with abnormalities due to the thyroid condition. There is also some reason to believe that in hypothyroidism, the conversion of carotene into vitamin A is impaired.

Analogues of Thyroxine.—With the view of relating structure to physiological action, several compounds closely related chemically to thyroxine have been prepared, and the biological action of such compounds has been tested. The diiodo compound, 3′,5′-diiodothyronine (1) (thyronine is the name given to thyroxine without its

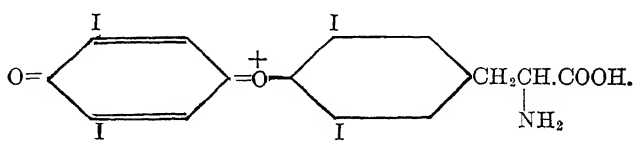
(1) 

iodine atoms) is but one-fourth as active as thyroxine. Of two isomers of thyroxine, the one with the OH group in the meta position (II) was inactive, and the other with the OH group in the ortho position (III) showed slight activity.

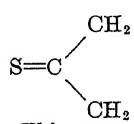
II 

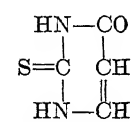
III 

Niemann suggests the necessity of a potential quinoid structure for the compound to show physiological activity (IV):

IV 

Thiourea and Thiouracil.—These two compounds, among several others, possess the property of inhibiting the production of thyroxine. This property has led to the use of these compounds in cases of hyperthyroidism.


Thiourea


Thiouracil.

Of the two, thiouracil is possibly the less toxic. Favorable results

have been reported in clinical cases, but bad effects have also been noted.

These substances and their uses have an interesting history. In the attempt to prevent the synthesis of some vitamins by intestinal flora, several investigators used sulfaguanidine. After several weeks a hypertrophy of the thyroid gland was noticed. The glands were several times larger than those of animals which had not received sulfaguanidine. There was at the same time a lowering of the basal metabolism. Administering iodine had no effect, but thyroxine reversed the process: the gland approached normality again and the basal metabolic rate was restored.

Many compounds were now tried in the attempt to duplicate the action of sulfaguanidine. Thiourea and thiouracil were picked as the most promising—the most potent, physiologically, with the least toxicity.

THE PARATHYROIDS

Attached to the thyroid are four small organs, the parathyroids, which for a long time were confused with the thyroid itself. The combined weight of the glands varies from 0.05 to 0.3 gm. The removal of the parathyroids causes tetany and a drop in the blood calcium. Clinically, tetany is characterized by "hyperexcitability of the nervous system which, when marked, causes intermittent tonic spasms of muscles. It represents a physiologic response of nerve and muscle tissue to certain disturbances in calcium metabolism."

Greenberg found that when rats were kept on a diet extremely low in calcium, tetany did not develop, even though the calcium in the blood was also extremely low. However, tetany did develop on diets low both in calcium and vitamin D.

Collip and Hanson prepared an extract, obtained by the acid hydrolysis of the gland, which when injected into a parathyroidectomized animal restored its health and, at the same time, raised the percentage of calcium in the blood. It was shown subsequently that the injection of a potent extract into a normal dog doubled the amount of blood calcium normally present (the normal amount being about 10 mg. per 100 cc.). The plasma calcium shows the first signs of an increase in about four hours, and then reaches a maximum in from twelve to eighteen hours; from then on a decrease sets in until the normal level is reached in from twenty to twenty-four hours.

The increase in plasma calcium is followed by an increased urinary excretion of calcium and inorganic phosphate and a decrease in blood phosphate.

It is believed that this extra calcium is derived from the bones by a withdrawal of the element. Under such conditions of hypercalcemia, the kidney undergoes pathological changes and abnormal deposits of calcium salts accumulate in soft tissues.

Parathyroid preparations have been used successfully in the treatment of tetania parathyreopriva (tetany caused by removal of parathyroids) and of infantile tetany. Occasionally, in operations on the

thyroid gland, tetany may develop due to removal of or injury to the parathyroid glands or their blood supply.

The hormone has not, as yet, been prepared in a pure condition, but the evidence points to its being a protein. The most potent preparations show an ultraviolet absorption spectrum almost identical with that of many simple proteins. Further, the action of pepsin completely inactivates the material.

Activated products of ergosterol (p. 184) and cholesterol also have the ability to increase serum calcium. Both vitamin D_2 (calciferol, p. 185) and dihydrotachysterol, the reduced product of tachysterol (p. 185), are used in parathyroid deficiency (hypoparathyroidism).

PITUITARY (HYPOPHYSIS)

This gland, "no larger than the end of the little finger," situated at the base of the skull, seems to have a multiplicity of functions. There are three distinct portions to this gland, the anterior, the pars intermedia and the posterior; and while all three may be important enough, it is the anterior portion which seems to be actually essential to life.

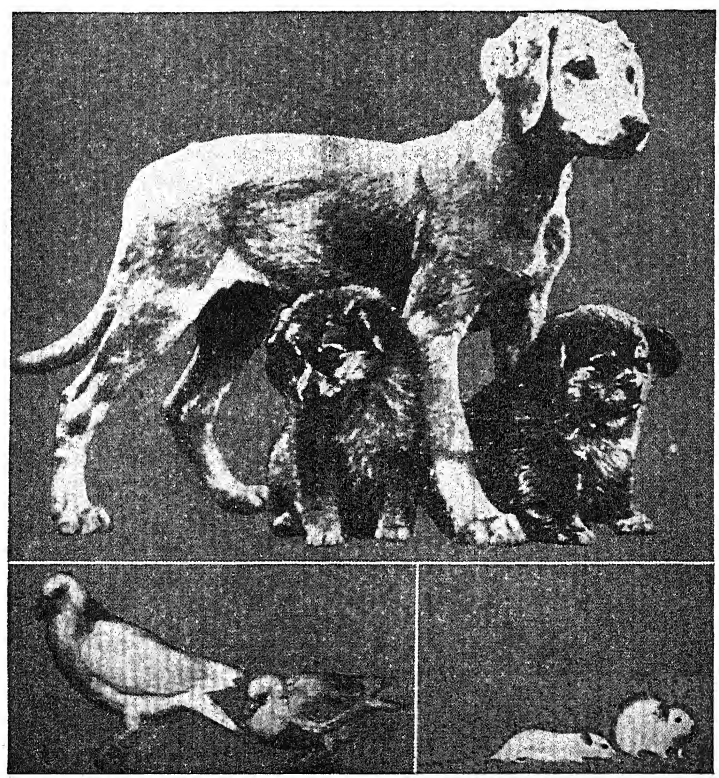


Fig. 117.—Effects of pituitary removal on body growth in animals. The two small pups were operated on when five days old and grew little thereafter though their litter mate is shown to have made good growth when all were photographed four months later (Kapran). This operation likewise stops growth in young pigeons and rats. (Riddle, *Scientific Monthly*, 47, 97.)

Clinically, types of gigantism and acromegaly (examples of a hyperpituitarism) and dwarfism (a possible example of hypopituitarism) have been known for some time. But more recently extracts have been obtained which show a multiplicity of actions. Since the active substances show the properties of proteins, the difficulties of separating them are great; and in many cases highly impure extracts are still used. It is still an open question as to whether the many effects obtained are due to the many hormones present, or whether but two or three hormones exhibit a number of different characteristics.

Fig. 118.—Sebastian de Morra. A painting by Velasquez. This is one of the most famous of the numerous paintings of dwarfs and endocrine types by Velasquez.

Anterior Pituitary.—We shall first take up the question of the anterior pituitary. There is evidence that this portion of the pituitary is responsible for the following: 1, growth hormone; 2, gonad-stimulating hormones; 3, lactogenic hormone; 4, thyrotropic hormone; 5, adrenalotropic hormone; 6, metabolic hormones.

After hypophysectomy the thyroids, the adrenal cortex and the gonads are much affected and their functional activity is greatly lessened. Lactation ceases. In general, atrophy of many of the endocrine glands and a deficient output of glandular secretions are evident.

While survival for a time is possible without the anterior lobe of the hypophysis, a normal life span is probably not possible.

In rats, as little as 10 per cent of the original gland will prevent the various deficiencies.

1. *Growth Hormone.*—Hypophysectomy in young animals gives rise to dwarfism and sexual infantilism (Fig. 117). Cretinic dwarfs

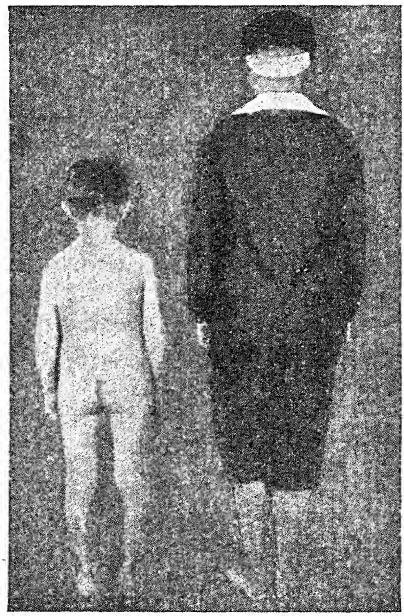


Fig. 119.—Pituitary dwarfism in boy aged thirteen and one-half years compared with a normal boy of the same age. The condition is due to insufficient growth hormone. (Courtesy of Dr. August A. Werner.)

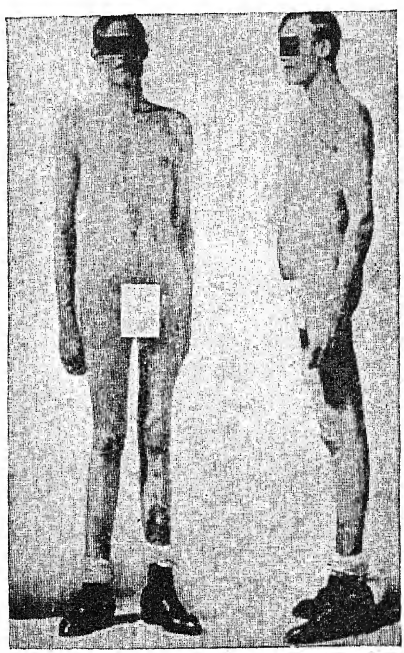


Fig. 120.—Pre-adult gigantism resulting from excessive secretion of the anterior pituitary growth hormone; age twenty-five years, height 7 feet 8.3 inches. (Werner, *Endocrinology, Clinical Application and Treatment,* Lea & Febiger, Publishers.)

may also be the result of a depression of the pituitary function (Figs. 118 and 119). Starting with alkaline extracts, Evans succeeded in preparing material which when injected into rats produced definite gigantism. "It is," writes Evans, "as if one could produce human beings of 10 or 12 feet."

Such growth appears to resemble normal growth and is not the result, for example, of an accumulation of water and fat. The hormone influences, in the main, skeletal growth by stimulating the epiphyseal cartilages; but it also affects the soft tissues, etc.

Cases of gigantism and acromegaly are shown in Figs. 120 and 121.

The extracts originally used by Evans were essentially dilute aqueous alkaline solutions which, in addition to containing the active growth hormone, retained varying quantities of a number of other

A B C D

Fig. 121.—A case of acromegaly. A, The patient at age twenty-four, before the onset of the malady; B, at age twenty-nine, at the time of onset; C, at age thirty-seven; and D, at age forty-two, when outspoken acromegalic changes are evident. (Cushing, *The Pituitary Body and Its Disorders*, J. B. Lippincott Co.)

hormones present in the anterior portion of the piuitary; but such extracts have been very much purified since then. In fact, Evans claims to have obtained the hormone in a chemically pure state. Assays were made on female rats hypophysectomized when twenty-seven days old. Injections were begun fourteen days later, once daily for ten days. 0.010 mg. of the purified product gave an increase of 10 gm. in body weight.

As evidence of the purity of the product, the injection of as much as five grams of the product failed to show the presence of any of the other hormones in the anterior portion of the pituitary (such as those dealing with lactogenic, thyrotropic, adrenocorticotropic and follicle-stimulating properties).

It should be pointed out that several investigators object to this conception of a "growth hormone"; for, as Smith points out, "growth is such a complex process, that it is difficult to conceive of its being due to a single hormone." However, that there is some "principle" or hormone in the hypophysis which is essential for general body growth seems well established.

2. *Gonad-Stimulating (Gonadotropic or Gonadotrophic) Hormones.* (See also p. 506.)—P. E. Smith and Aschheim and Zondek discovered that when a piece of anterior pituitary tissue is implanted under the

skin of an immature rat, the ovaries develop within a few days. Zondek later postulated that the growth of the ovarian follicles is due to a "follicle-stimulating hormone" ("prolan A"), and the development of lutein tissue, to a "luteinizing hormone" (see p. 512). The follicle-stimulating hormone is also called "thylakentrin," and the luteinizing hormone, "metakentrin."

Thylakentrin and metakentrin have been separated from one another (the latter is not soluble in one-third saturated ammonium sulfate solution at *p*H 7.5 while thylakentrin is soluble). Thylakentrin has been sufficiently purified so that if contains at best negligible quantities of other pituitary hormones. Even purer products of metakentrin have been obtained, showing homogeneity in electrophoretic and ultracentrifugal studies. The material isolated from sheep shows a molecular weight of about 40,000, an isoelectric point of 4.6, a carbohydrate content of 4.5 per cent and tryptophan to the extent of 1 per cent.

Gonadotropins other than those obtained from the pituitary are known. These substances may be classified as follows: (1) Human chorionic* gonadotropin (present in blood, urine and tissues of pregnant women); (2) human non-chorionic gonadotropin (present in blood and urine of ovariectomized and post-menopausal women); and (3) equine gonadotropin (present in blood and placental tissue of the pregnant mare). There is evidence which indicates that extracts of human placenta, pregnancy blood and urine contain gonadotropic substances which are not the same as the one in the pituitary. However, pregnant mare's serum contains a substance more comparable to the gonadotropins in the pituitary and different from human placental gonadotropin hormones.

Some of these substances have been prepared in a high state of purity. They are proteins containing galactose and hexosamine.

Zondek and Aschheim originally discovered a gonadotropic hormone in the urine of pregnancy, which led them to their now well-established pregnancy test. The principle involved in the pregnancy test is to inject immature mice with the suspected urine, kill the animals on the fourth day and examine the ovaries for hemorrhagic spots and yellowish protrusions (developed corpora lutea) (p. 506).

During pregnancy, we find a relative abundance of an estrogenic (female) hormone and the gonadotropic hormone in the urine. The latter appears in recognizable quantity by the first missed period (the Aschheim-Zondek test), and reaches its maximum between the second and third months of pregnancy; the former appears somewhat later, but lasts until birth, and then decreases very rapidly (p. 506).

3. *Lactogenic Hormone.*—Riddle was among the first to prepare an extract of the pituitary which stimulates the enlargement and functioning (formation of "crop milk") of the crop glands in pigeons (Figs. 122 and 123). This hormone, which initiates lactation, is known as *prolactin, mammotropin* or *galactin.*

It is believed that a female hormone produced by the placenta

* Pertaining to the more external of the two fetal membranes.

during pregnancy stimulates the growth of the mammary gland and at the same time inhibits the secretion of prolactin. At parturition, the inhibiting influence of the placenta is removed, the prolactin is released, and the secretion of milk is fostered.

One method of preparing active extracts has more general possibilities and the principle involved will be given. If pituitaries are shaken with chloroform at a *p*H of 5 to 6 and then centrifuged, three layers are obtained. The lowest, or chloroform layer contains most of

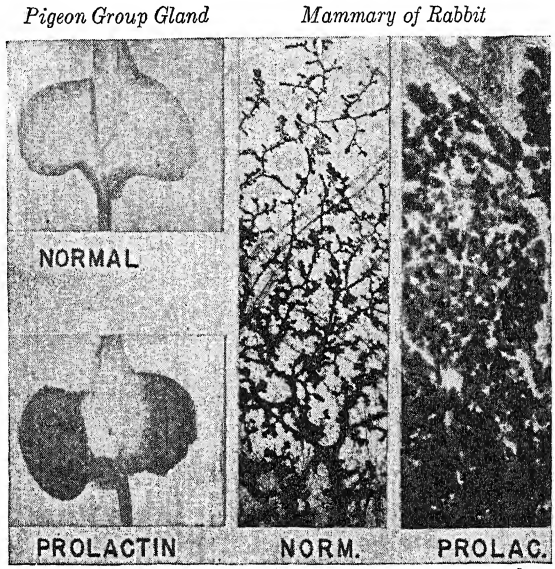


Fig. 122.—Showing the action of prolactin on crop sacs and on mammary gland. When unstimulated by prolactin, the walls of the pigeon's crop are very thin and transparent. But either the release of prolactin by the bird's own pituitary or injection of prolactin from a hen, a calf, or a whale causes the lateral pouches of the crop wall (and these parts only) to thicken greatly and produce "pigeon milk." After the cells and ducts of rabbit mammary glands have developed properly from two to four injections of prolactin will cause them to form and store milk. Other pituitary hormones do not stimulate milk secretion. (Riddle, *Scientific Monthly*, **47**, 97.)

the lipid substances. The layer above the chloroform is a gel and contains most of the tissue porteins, including prolactin and adrenotropic hormone (p. 490). The top layer, an aqueous solution, contains, among others, the gonadotropic hormones, thyrotropic hormone (p. 489) and pituitrin (p. 492).

The prolactin is extracted from the chloroform gel by extraction with acidulated methanol and fractionation with sodium chloride.

Crystalline products have been obtained by Evans, White and Riddle. The molecular weight is said to be in the neighborhood of 25,000. The sulfur content is from 1.8 to 2.0 per cent. Tyrosine, tryptophan, cystine, methionine and arginine are among the amino acids present.

4. *Thyrotropic Hormone.*—An appropriate extract of the pituitary injected into normal animals results in the enlargement and hyper-

plasia of the thyroid. There is an increase in the metabolic and in the heart rate and the development of an exophthalmos resembling Graves' disease. Smith had been among the first to show that the extirpation of the pituitary results in the atrophy of the thyroid. This condition can be improved by implanting fresh pituitary into a hypophysectomized animal.

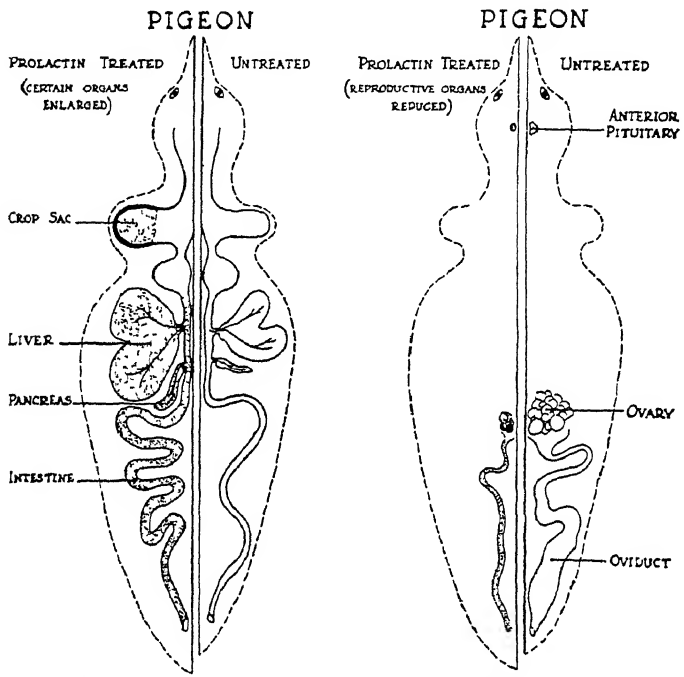


Fig. 123.—The gross structural changes induced in pigeons by prolactin. Prolactin increases the weight or size of the body as a whole and tends to induce growth in several parts of the digestive apparatus—crop sacs, liver, intestine, pancreas. It decreases the output of follicle-stimulating hormone from the pituitary gland, and in this indirect way it causes a pronounced atrophy of the testis, ovary, and oviduct. (Riddle, *Scientific Monthly*, 47, 97)

Active extracts injected into rabbits or guinea-pigs—rats are more immune—results in symptoms of exophthalmic goiter: the thyroid is increased in size, the iodine is decreased, there is a loss of colloid and the cells are enlarged, or hypertrophied. There is also a rapid rise in basal metabolism (p. 411).

Several claims have been made in reference to the isolation of the hormone. It appears to be a protein belonging to the globulin group and is somewhat more soluble in water and salt solutions than some of the gonadotrophins.

Radioactive iodine studies have suggested that this hormone is involved in the rate of uptake of iodine in the thyroid and also in the conversion of diiodotyrosine to thyroxine.

5. *Adrenotropic (Corticotropic) Hormone.*—Hypophysectomy results in the atrophy of the adrenal cortex. Improvement is possible by

using pituitary implants or by injecting appropriate pituitary extracts; but no improvement results when the cortical hormone is injected. It is postulated, therefore, that the anterior lobe of the pituitary manufactures an adrenalotropic hormone.

White and Long, and Evans have obtained products which are probably pure.

For his assay method, Evans has used the repair of the adrenal cortex of the immature female rat, 26 to 28 days of age at hypophysectomy. The increase in width of the cortex and redistribution of the lipids were the criteria. 0.05 mg. of the final product was enough to cause detectable repair.*

6. *Metabolic Hormones.*—Particularly since the discovery of insulin, it has been believed that blood sugar was regulated primarily, if not exclusively, by the pancreas. It has already been pointed out that the removal of the pancreas leads to diabetes. However, Houssay showed that if the pituitary is also removed, the rise in blood sugar can be prevented. Both Houssay and Evans have since shown that the injection of anterior pituitary extracts into normal animals produces a hyperglycemia and a glycosuria.

Largely through the work of Young, it has been established that the anterior pituitary contains a "diabetogenic hormone" which causes a rise in blood-sugar level. But even more arresting is the observation that by increasing the daily dose of the pituitary extract (equivalent to 25 gm. of fresh ox anterior lobe), and then stopping the injections, the diabetic condition continues and presumably becomes permanent (Fig. 124). This is true—in many cases but not in all—of the dog; but it apparently does not apply to the cat.

In addition to a diabetogenic hormone, which tends to increase the amount of sugar in the blood, there is an "insulinotropic substance"—there is some debate as to whether we are dealing with a hormone—which stimulates the production of insulin.

Dogs made permanently diabetic with the diabetogenic hormone exhibit injury of the islands of Langerhans in the pancreas (the seat where insulin is manufactured); and it is possible that some cases of diabetes in man may be due to an overactivity of the hypophysis.

In addition to affecting carbohydrate metabolism, Funk and others are of the opinion that the pituitary also harbors a "fat-metabolism" hormone. Extracts have been obtained which, when injected, cause a very marked increased in the acetone body production.

Extracts from urine have been obtained which have a hyperglycemic function and which can also give rise to acetone bodies. It is not clear whether such active material has its origin in the pituitary (Harrow).

The preparations of those various extracts is still in an unsatisfactory state. They involve various acid and alkaline extracts, sometimes fractional precipitations, sometimes adsorption procedures, sometimes delicate *p*H adjustments, etc.

* For the method used by Evans in the preparation of his extract, see appendix, p. 572.

Posterior Lobe of the Pituitary.—Aqueous extracts of this portion of the gland (pituitrin) have long been used in labor. Two substances have been separated by Kamm and others: one, *pitressin*, raises blood pressure and checks the secretion of urine; the other, *pitocin* (*oxytocin*), contracts the muscles of the uterus and perhaps functions during parturition to remove the products of conception.

Pitressin stimulates the peripheral blood vessels and causes a rise in blood pressure. It has been used to combat the low blood pressure of shock following surgery. The claim has been made that, under certain

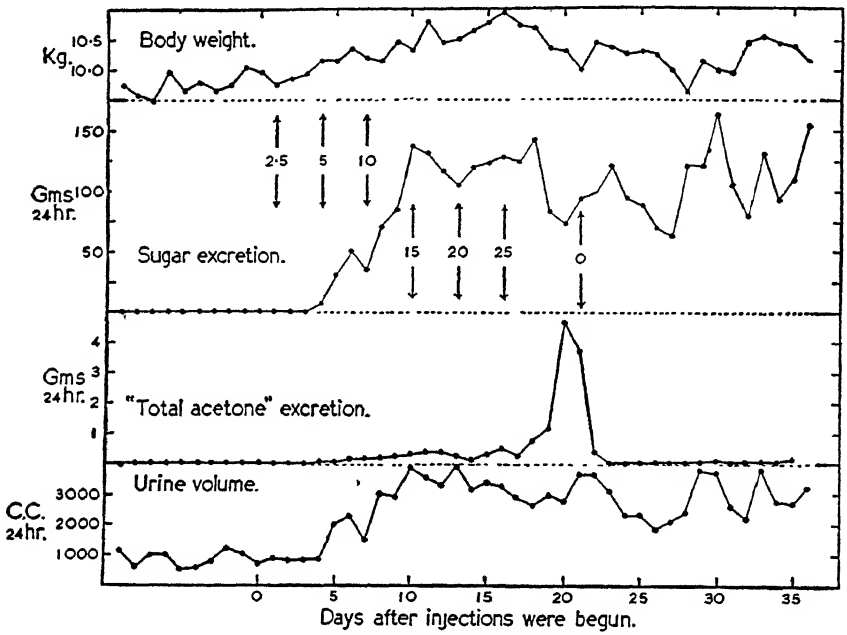


Fig. 124.—Data relating to dog 44 during the period of establishment of the permanent diabetes. The figures on the arrows give the weight in grams of fresh ox anterior pituitary tissue used to prepare the amount of crude extract injected daily, from the day indicated by the arrow, onward. The injections ceased on the day indicated by the arrow marked "o." The ketonuria fell to a low level following the cessation of injections but rose later. (Young. *New England J. Medicine*, **221**, 635.)

conditions, pitressin has the advantage over epinephrine in that the former gives rise to a more gradual increase in blood pressure, and that this pressure is of longer duration.

It has been shown that the injection of pitressin in cases of diabetes insipidus—a disease characterized by the elimination of large quantities of urine—checks the flow of urine. For example, in a typical case a patient, before treatment, had taken about 7200 cc. of fluid during twenty-four hours (great thirst is a symptom) and had eliminated 9550 cc. of urine, which is from five to six times the normal quantity. After treatment with pitressin, the fluid intake dropped to 3000 cc., and the urinary output to 3700 cc.

By means of electrophoretic studies, using the principle of electrical

transport and the relative migration velocities of different molecules, du Vigneaud and his associates have shown that starting with the juice of the posterior gland, "the pressor activity travelled at a faster rate than the oxytocic activity, thus demonstrating that the activities . . . were manifestations of different chemical entities."

The evidence points to the pressor and oxytocic substances as polypeptides, with molecular weights ranging between 600 and 2,000.

Du Vigneaud has also isolated a protein in an apparently pure condition which shows pressor, oxytocic and antidiuretic activities; these activities "are inherent properties of the molecule."

Pars Intermedia.—According to Zondek, this lobe of the pituitary manufactures a hormone which can be recognized by its effect on the pigment cells of the skin of lower vertebrates. The injection of an extract into a minnow (*Phoxinus laevis*) causes the development of a red color at the point of attachment of the thoracic, abdominal, and anal fins. Beyond its exerting an influence on the chromatophores of cold-blooded animals, the significance of this hormone, called *intermedin*, is not clear.

INSULIN (See also p. 315)

As has already been stated, the pancreas has two distinct functions: it secretes a juice (pancreatic juice) which flows into the intestine and which contains digestive enzymes; and it secretes a hormone, insulin, which finds its way into the blood and which plays an important rôle in the regulation of carbohydrate metabolism.

The removal of the pancreas in an animal gives rise to diabetes: the sugar in the blood increases (hyperglycemia), sugar appears in the urine (glycosuria), and acetone bodies are excreted. Death results in about three weeks. Banting, Macleod, Best, and Collip prepared an acid alcoholic extract of the pancreas which prevented these symptoms in a pancreatectomized animal, and which could be used to relieve diabetic sufferers among human beings. The hormone is called *insulin*.

When injected into normal animals, insulin lowers the blood sugar and finally convulsions occur. This discovery led to a method for standardizing the hormone.

One method—and there are many—of preparing active extracts containing insulin is to extract the pancreas with dilute and acidified alcohol and to precipitate the hormone by the addition of ammonium sulfate. Further purification is obtained by isoelectric precipitation of insulin at *p*H 5.

Starting with highly active commercial fractions, Abel and his coworkers obtained crystalline insulin by further purification with pyridine, phenol, and brucine.

Crystalline preparations of insulin all contain zinc. In any case, apart from the zinc, the hydrolysis of insulin yields nothing but amino acids, among which cystine and glutamic acid are prominent. Tryptophan has not been detected (Table 81).*

Proteolytic enzymes attack insulin; and, indeed the hormone has

* It may be significant that normal pancreatic tissue is relatively rich in zinc.

to be injected rather than be given by mouth. It has been claimed that during peptic hydrolysis of insulin, the decrease in its activity runs parallel with a decrease in its tyrosine content.

Insulin is easily destroyed by alkali but is relatively stable in slightly acid solutions.

Stern and White acetylated insulin with ketene, $CH_2=C=O$. They found that when ketene acted on insulin for five minutes at room temperature and at *p*H 5.7, only free amino groups were acetylated. If the reaction is continued beyond this time, the hydroxyl groups of tyrosine are slowly acetylated. In this way, it could be shown that acetylating the free amino groups of insulin has no appreciable effect on its activity; but, on the other hand, when the hydroxyl groups on tyrosine were acetylated, there was marked reduction in the activity of the hormone.

These results are of special interest, since Northrop and Herriott showed that the tyrosine group plays an analogous rôle so far as the activity of pepsin is concerned.

TABLE 81.—ANALYSIS OF CRYSTALLINE INSULIN (White and others).
Molecular weight, 35,100
Isoelectric point, *p*H 5.35

Amino acids.	*Per cent*
Leucine	30
Tyrosine	12
Arginine	3
Histidine	4
Lysine	2
Glutamic acid	20
Cystine	12 5
Proline	10
Phenylalanine	present
Methionine	?
Threonine	2 6
Serine	3 6

(Zinc is present to the extent of 0.52 per cent. The percentage of sulfur amounts to 3 3.)

Insulin Protamine, Etc.—A notable advance in insulin therapy was made by Hagedorn and Jensen who, by combining insulin with protamine (one of the simpler proteins), prepared a product which, when injected, is absorbed more slowly than insulin itself, and whose effects are therefore more lasting. Instead of two and three injections a day, often but one suffices. The addition of zinc to protamine insulin was suggested by Scott and Fischer. This "protamine zinc insulin" prolongs the effective action of insulin; it lowers the blood sugar for more than twenty-four hours.

Globin insulin with zinc has also come into use. The "globin" is the protein derived from hemoglobin. The "action" time is intermediate between that of insulin alone and protamine zinc insulin. This globin insulin with zinc is a "twelve to fifteen hour insulin" (the action lasts that long) and in certain cases, involving careful regulation of diet, is preferred.

In the use of protamine zinc insulin, a drop in blood sugar begins in four to six hours; with insulin alone, the action is immediate; with globin insulin and zinc, the lowering occurs within two hours.

Insulin "Shock."—Another advance we owe to Sakel, who finds that insulin injections almost up to the point of "shock" often have a beneficial effect in certain mental disorders (schizophrenia or dementia praecox).

Insulin and the Pituitary.—The pituitary (p. 484) and possibly the adrenals are involved in the activity of insulin. "The diabetic state may not be due primarily to subnormal secretion of antidiabetic hormone, but to various other hormonal disturbances . . . " (Waters and Best).

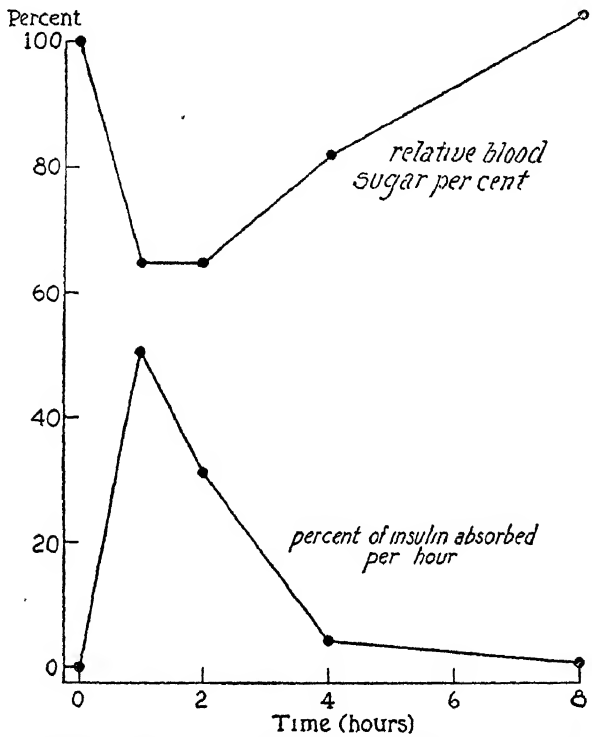


Fig. 125.—Comparison of the rate of absorption of radio-active insulin with blood sugar levels. (Reiner, Keston and Green, *Science*, **96**, 362.)

Absorption of Insulin.—By the incorporation of radioactive iodine —obtained by the deuteron bombardment of tellurium—in iodo-aniline, diazotizing the product and coupling with insulin in the form of an azo derivative, a study could be made of the absorption and distribution of insulin labelled with a radioactive element (which could always be identified by measurements in a Geiger counter).

In Fig. 125 is shown the relation between the rate of absorption of radioactive insulin and the blood sugar at various times following the injection: The time of maximum absorption coincides with a maximum drop in blood sugar. An hour after injection, the circulating blood contains a considerable quantity of radioactive material.

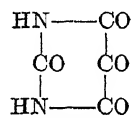
Relatively speaking, the concentration of radioactive material is particularly great in the liver and kidneys.

Mechanism of Insulin Action.—Very little is known concerning the mechanism of insulin action: just how it plays its part in carbohydrate metabolism; how it brings about an increased deposit of glycogen in striated muscle; how it brings about the oxidation of glucose.

Since phosphorus compounds are intimately involved in these changes, experiments using the radioactive isotope (P^{32}) have been carried out. The injection of insulin results in an increased "turnover rate" of phosphocreatine and of two of the phosphate groups (label phosphate groups) of adenosine triphosphate, during glucose absorption. This confirms the prominent part phosphorus compounds play in the process.

While there is an increase in the storage of sugar as glycogen under the influence of insulin, less than 25 per cent of the extra sugar which is retained is stored in this way. Drury is of the opinion that during this transient stage of storage when under the influence of insulin, much of the carbohydrate is stored as fat.

Alloxan Diabetes.—Alloxan, a substance structurally related to uric acid (p. 85) and riboflavin (p. 153)—part of the molecule of the latter has an alloxan configuration—produces diabetes in various animals. A single injection will often produce the disease in 24–28 hours.



Alloxan.

The disease is brought about by the destruction of pancreatic tissue (selective necrosis of the islets of Langerhans) with a lessening production of insulin.

While it is also possible to produce experimental diabetes in the animal by the injection of the diabetogenic hormone from the pituitary (p. 491) or by partial pancreatectomy, the diabetes so obtained is considered as due to "overwork" of the beta cells; alloxan diabetes brings about their actual destruction. As evidence of this view, in the early stages of the disease, starvation or treatment with insulin will prevent the diabetes due to surgical operation or the injection of the anterior pituitary extract; but this is not true of diabetes due to alloxan.

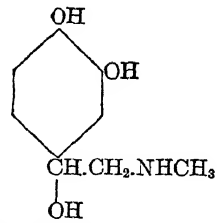
THE ADRENALS

There are two distinct parts to the adrenals, the medulla and the cortex. The medulla, an offshot of the sympathetic nervous system, contains the hormone epinephrine; and the cortex, essential to life, contains several hormones which have been isolated.

Epinephrine, or *adrenaline,* was first isolated by Abel and Taka-

mine. The principle employed by Takamine in isolating the hormone was to extract the glands with warm acidulated water, filter, concentrate the filtrate, precipitate inert material with an excess of alcohol, and finally precipitate the adrenaline itself with ammonia.

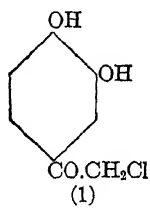
Its formula is:



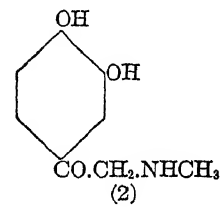
Epinephrine or adrenaline.

which means that it is catechol to which a hydroxyethylmethylamine group is attached.

One method of synthesizing the substance is to combine catechol and chloracetylchloride (a mixture of chloracetic acid and phosphorus oxychloride) to form chloracetocatechol (1). (1) combines with a concentrated solution of methylamine in the cold to form the methylamino ketone (2), which, on reduction with hydrogen and palladium, yields epinephrine.



(1)



(2)

Epinephrine constricts the splanchnic and cutaneous blood vessels, causing a rise in blood pressure; it accelerates the heart rate; it causes a temporary increase in blood sugar and blood lactic acid.

The *l*-form is fifteen times more effective than the *d*-form.

Epinephrine is used in shock and collapse, in asthmatic attacks, and in combination with local anesthetics (to prevent bleeding).

Epinephrine is the most powerful vasoconstrictor known. It is this property which makes it so useful as an adjunct in local anesthesia. The anesthetic effects are prolonged by adding epinephrine to the solution of the local anesthetic to be used. Not only does the hormone prolong the anesthetic effect, but less of the anesthetic is needed.

Cannon is of the opinion that one function of epinephrine is to act in emergencies (in cold, fatigue, shock, etc.).*

* "The adrenal medulla cooperates with sympathetic impulses in producing adrenaline. This sympathico-adrenal system is brought prominently and usefully into action in emotional excitement, in vigorous muscular work, in asphyxia, low blood pressure, chilling surroundings and hypoglycemia—in brief, it serves effectively in emergencies; furthermore, this service can be given a general expression in stating that the system guards the constancy of the internal environment of the organism; and finally that secreted adrenaline itself acts to prolong the effects of nerve impulses, to accelerate metabolism, to shorten coagulation time and to

It has already been mentioned (p. 314) that epinephrine plays a rôle in the metabolism of carbohydrates. Its effect is to increase the conversion of liver glycogen to blood sugar and to increase the conversion of muscle glycogen to lactic acid (which is ultimately converted to liver glycogen) (Fig. 126).

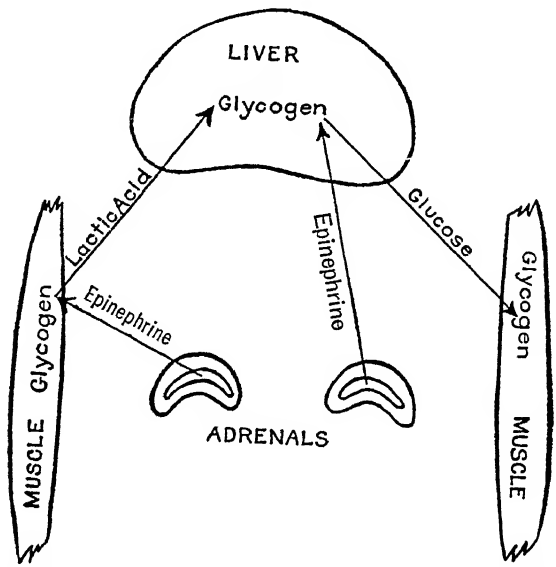


Fig. 126.—Effect of epinephrine on carbohydrate metabolism. (Hartman, *Ohio J. Science*, **37**, 427)

Cortical Hormone (Cortin).—Swingle, Hartman, and others prepared extracts which prolonged the life of adrenalectomized animals. The principle of one method of preparation employed by Swingle was to extract the glands by means of a mixture of alcohol and benzene, filter, concentrate the filtrate, add acetone (which takes up the active material), and purify still further by selective distribution between 70 per cent alcohol and petrôleum ether, the hormone passing into the former.

The average age-span of an adrenalectomized cat is from seven to eight days. Such cats have been kept alive for more than a year by appropriate treatment. A number of sterol-like substances have been isolated from extracts (Reichstein; Kendall; Wintersteiner). The formulas for several related substances are given (Fig. 127).

These substances have varying biological activity. For example, desoxycorticosterone is some six times as active as corticosterone. But merely speaking of "activity" is not enough without further specification. For instance, some of the compounds are particularly effective in the formation of carbohydrates, and the efficiency of muscles, while others affect mainly renal function and the distribution of water and electrolytes.

release glucose from the liver. There is no evidence that secreted adrenaline is an important agent in maintaining a high blood pressure" (Cannon).

Thorn has shown that 17-hydroxycorticosterone, a compound active in its effect on carbohydrate metabolism, has no effect on sodium retention in the dog. On the other hand, desoxycorticosterone is markedly effective in causing sodium retention, while its effect on carbohydrate metabolism is little, if any (Fig. 128).

While the name *cortin* has been given to the potent extract, we now know that cortin is not really one substance, but represents several substances of varying activity.*

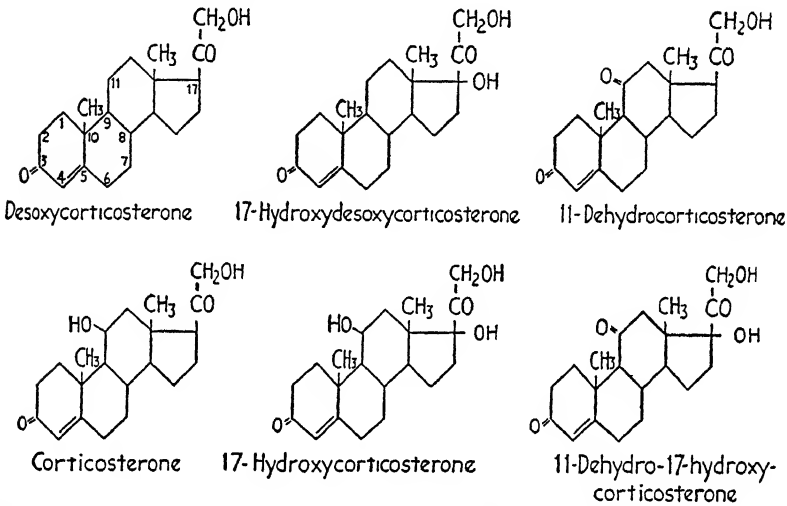


Fig 127.—Steroids with known physiological activity isolated from adrenal cortical tissue.

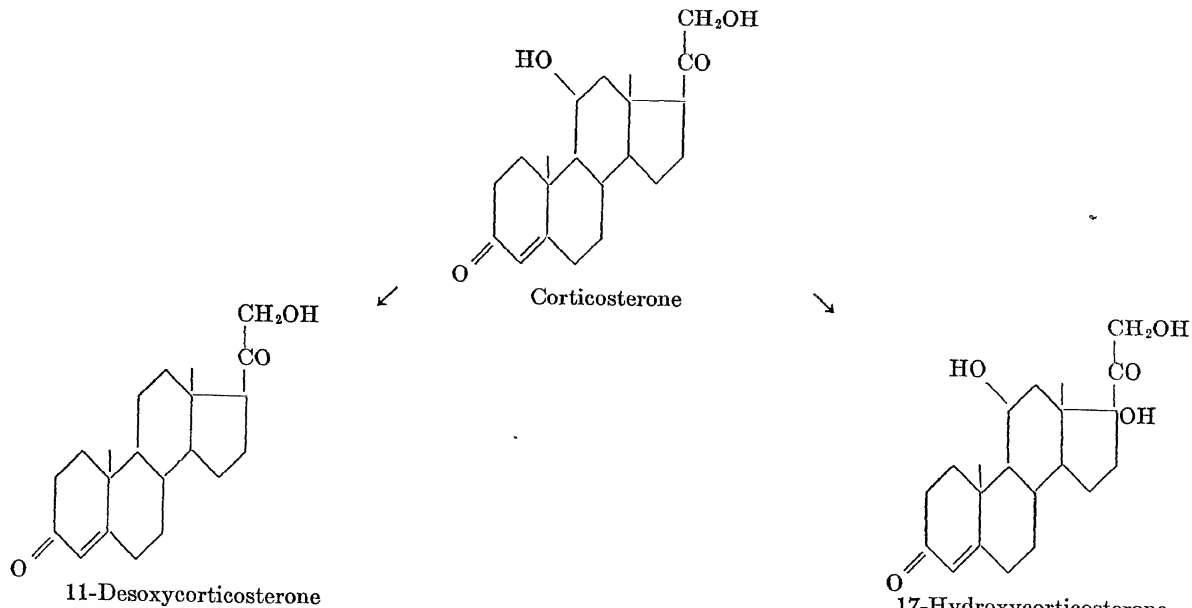
Addison's Disease.—This disease, associated with a degeneration of the cortex, has been successfully treated with extracts of the adrenal glands, and with desoxycorticosterone (p. 499), one of the hormones of the gland which has been synthesized.

Desoxycorticosterone is but one of a number of substances isolated from the adrenal cortex (see Fig. 127); in fact, some twenty odd have so far been isolated. It is the most potent of the group; but apparently it cannot be regarded as a complete substitute for the adrenal cortex in the sense that insulin may be a substitute for the internal secretion of the pancreas. For example, in Addison's disease, or in adrenalectomized animals, there are various metabolic disturbances—water, inorganic salts, carbohydrate, etc. Desoxycorticosterone will remedy some but not all of these conditions. It will cause the retention of sodium and water, but it has little effect in maintaining normal carbohydrate metabolism.†

Hartman believes there are two essential "factors" in the adrenal cortex: *cortin*, a "factor" or extract which enables an adrenalectomized

* The name "adrenal cortex extract" is now generally used.

† Pregnanediol, a product of the excretion of the corpus luteum hormone (page 512, has also been found in the urine after the oral administration of desoxycorticosterone (as its acetate) to patients with Addison's disease, and to an ovariectomized chimpanzee.



NaCl— Retaining effect	++++	++	0
CHO— Regulating effect	0	++	++++

Fig. 128.—Adrenal steroids: relationship of chemical configuration to physiologic activity. (Thorn, *J. Am Med. Assoc.*, **125**, 11.)

animal to maintain health, despite low plasma sodium; and the other, the "sodium factor," which is primarily responsible for sodium retention.

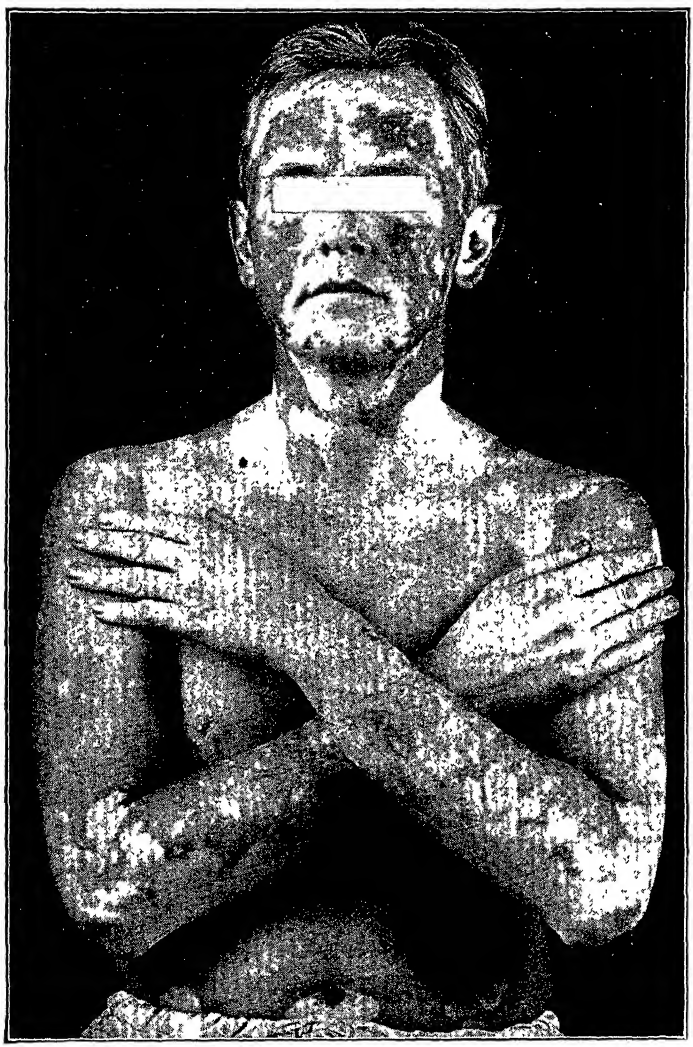


Fig. 129.—The presence of both pigmentation and depigmentation in a patient with Addison's disease. (R. F. Loeb, *Bull. N. Y. Acad. Med.*, June, 1940, p. 347.)

"Addison's disease is characterized in most cases by the insidious development and progression of asthenia [lack of strength] and fatiguability. . . . The symptoms are usually accompanied by the appearance of brownish pigmentation which increases over a period of months and which may be blotchy in its distribution or generalized. . . . " (R. F. Loeb) (Fig. 129).

The pigmentation which is often, but not invariably, present may be due to a disturbed series of enzyme reactions. It is apparently specific in man, for pigmentation has not been observed in adrenalectomized animals.

Adrenalectomized Animals.—So long as adrenalectomized animals are treated with adrenal extract they remain in presumably good health. However, when the extract is withdrawn, the urinary excretion of sodium is increased and its concentration in blood decreased. The

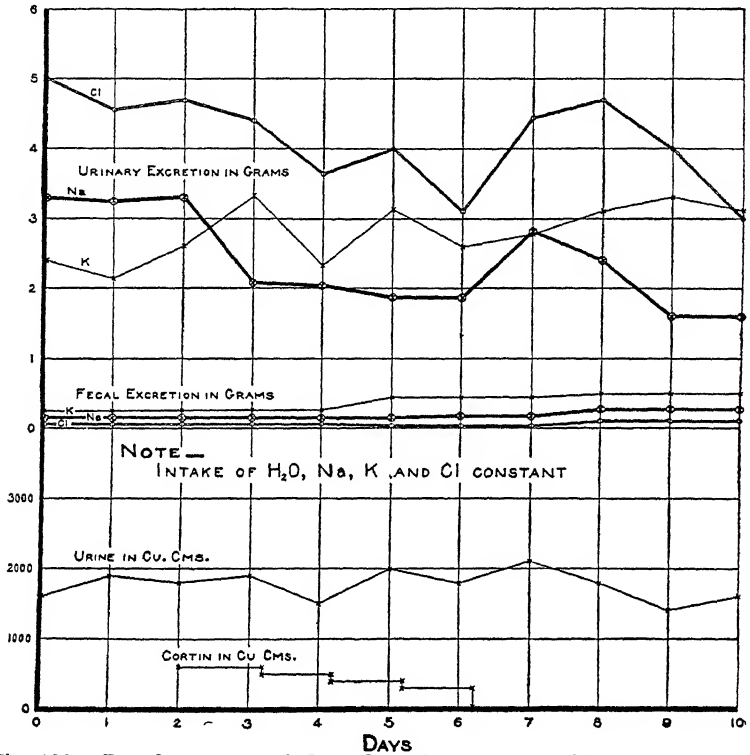


Fig. 130.—Renal excretion of electrolytes in a case of Addison's disease. Note that Na and Cl excretions are reduced while the K excretion is increased after cortin injections. (Hartman, *Ohio J. Science*, **37**, 427.)

plasma chloride and bicarbonate are also reduced, but the quantity of potassium is increased. Further effects of the withdrawal of the extract is loss of water from blood and tissue spaces, which leads to loss of weight and to a state of dehydration. The reinjection of the active material restores the quantity of electrolytes in the blood, and the dehydration disappears (Figs. 130, 131).

An interesting observation is that the administration of potassium salts to an adrenalectomized animal aggravates the condition; but when enough sodium chloride and sodium citrate, and very little potassium salts are given, the animal improves markedly. Under such treatment the amount of the extract of the adrenal cortex necessary to maintain the animal in a healthy state is considerably less than

without the sodium treatment. Ultimately it may be possible to maintain normal conditions without the use of the extract at all.

R. F. Loeb was among the first to show that the concentration of sodium in the blood of patients suffering from adrenal cortex insufficiency was decreased. He also observed that the addition of sodium salts relieved the condition.

That sodium salts—or rather the sodium ion—were vitally concerned with this disease was strikingly shown by withdrawing sodium ion from the diet of patients with Addison's disease; the condition of the patients promptly becomes worse (Harrop).

In adrenal cortex insufficiency there is also a disturbance in water metabolism: there is a tendency, for a time at least, for water to be lost.

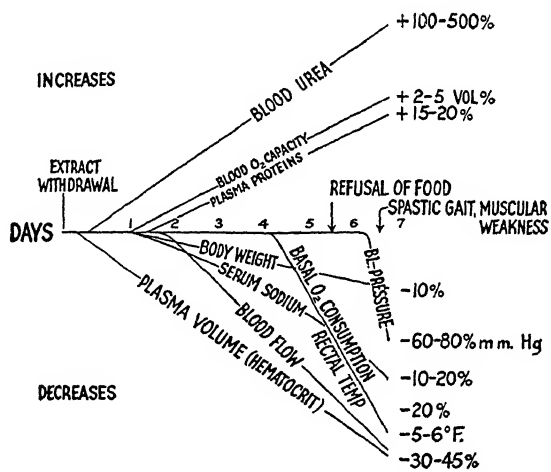


Fig. 131.—Chart showing graphically the onset of symptoms of cortical insufficiency following withdrawal of cortical extract from a group of adrenalectomized animals previously maintained in good condition by hormone administration (Modified from Harrop, Weinstein, Soffer, and Thrascher: *Jour. Exp. Med.*, **58**, 1933; *Therapeutic Notes*, March, 1936, p. 65, by courtesy of Parke, Davis & Co.)

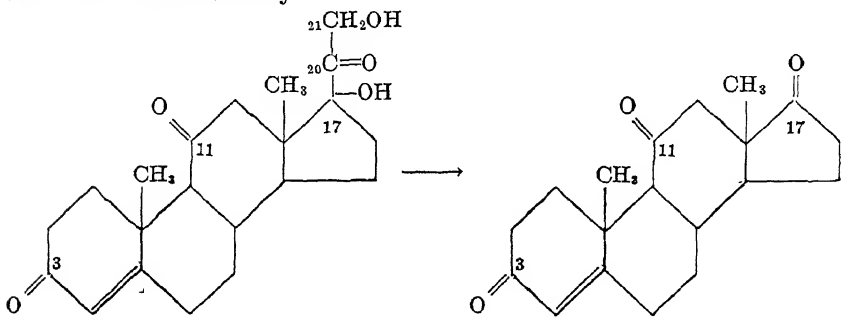
In a general way, as the sodium is lost in this disease, potassium tends to increase; as more sodium is excreted, less potassium is excreted.

Adrenals and Sex.—The cortex of the adrenals also elaborates substances belonging to the group of sex hormones.* Abnormal changes in sex may sometimes be due to disturbances in the adrenal cortex involving sex hormones. The female assumes male secondary sex characteristics and at the same time certain female characteristics become repressed. This is a type of masculinity or virilism known as "adrenal virilism." The cases are less frequent in the male; but when they do occur, the tendency is towards feminization—enlargement of breasts, genital atrophy, etc.

* The following paragraphs might be read in conjunction with the section dealing with the sex hormones (p. 505).

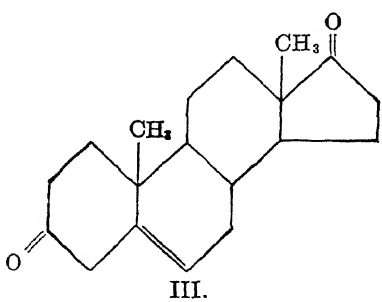
These developments are due, first, to the fact that the adrenals manufacture androgens and estrogens, male and female hormones (which is, apparently, a perfectly normal function); but, second, that due to some unknown cause, the hormones may be produced in excessive amounts, or their normal metabolism may be disturbed—in any case, giving rise to these sex changes.

Reichstein and others have isolated several compounds from beef adrenals which are androgens or male hormones (p. 514). One of them, adrenosterone (I) shows a capon comb test (p. 513) equivalent to one-fifth that of androsterone (p. 514). Compound I has been obtained artificially from 11-dehydro-17-hydroxycorticosterone (II), a compound which has already been included among the cortical hormones (p. 499) and which is effective to some extent in maintaining life after adrenalectomy.



It is also of interest to note that often malignant cortical tumors (adrenal carcinoma, for example) give rise to the excretion in excessive quantities of androgens; and this irrespective of the sex of the patient.

One of a number of these androgens isolated from the urine is dehydroisoandrosterone (III), a compound which is excreted in small



amounts by normal men and women and which, even here, probably means that its source is in the adrenals rather than in the reproductive organs.

From such observations it seems reasonable to conclude that not all of the androgens excreted by normal subjects have their source in the gonads, but some of them, at least, are derived from the adrenal

cortex. It is significant that the urine of eunuchs and of ovariectomized women still shows the presence of androgens.

High amounts of estrogens, or female hormones, are sometimes excreted in cases of virilism. Here, too, it may be noted that the urine of male and female castrates shows some estrogenic activity, suggesting the adrenal cortex as the origin of such substances. The typical estrogen, estrone or theelin (p. 508) has actually been isolated from adrenal glands.

Are the compounds in the adrenals which are related to the sex compounds merely by-products of truly cortical substances manufactured by the gland? Or are they specifically manufactured by the adrenal cortex to help regulate normal sex functions? We cannot answer these questions as yet.

THE SEX HORMONES (See also p. 503)

Under the stimulation of hormones from the anterior pituitary (gonadotropic hormones), the sex hormones in the testes and in the ovary are formed.

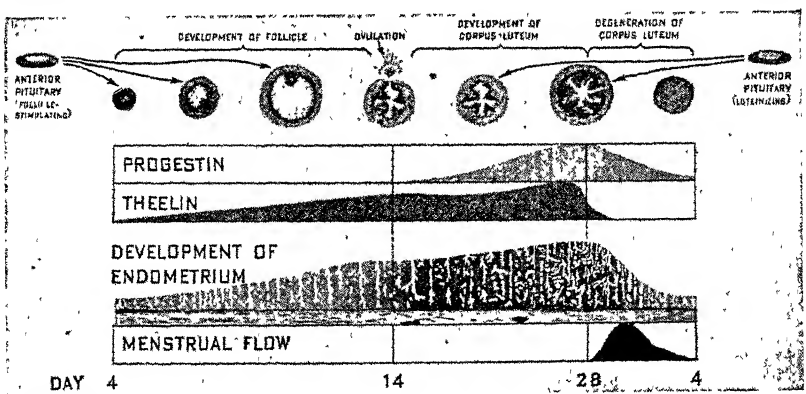


Fig. 132.—Normal menstrual cycle. Rapid regeneration of endometrium (following menstrual flow of previous cycle) under the influence of theelin, elaborated by the developing follicle (4th to 14th day)—ovulation (14th day) followed by slight decrease in theelin—steady increase in amounts of theelin and progestin during development of the corpus luteum (15th to 28th day); under the influence of progestin the highly vascular endometrium is changed to the secretory type (these changes are considered essential to subsequent rapid destruction of the endometrium following withdrawal of progestin)—sudden degeneration of the corpus luteum (if conception does not occur) and withdrawal of theelin and progestin (28th day)—rapid destruction of the endometrium and subsequent sloughing (1st to 4th day of new cycle). (*Therapeutic Notes*, courtesy of Parke, Davis & Co.)

The genital tract and the accessory male organs are influenced by the male hormone. One method of detecting the presence of an active extract is to observe its effect on the growth of comb and wattles in a capon. The cocks are castrated; this is followed by a shrivelling of the comb and wattles. The injection of a potent extract causes a renewal of growth of these secondary sex organs (Funk and Harrow; Koch).

In the female, two types of hormones are found to function. One type, as represented by estrone (theelin), is a product of the ovary;

the other, progesterone (progestin), is derived from the corpus luteum, which is formed after the ovum is ruptured and expelled. Both hormones control the uterine cycle.

Under the activation of the hormones from the anterior pituitary (the sex-stimulating hormones or gonadotropins), the ovary elaborates "female hormone" (estradiol) which causes the endometrium—the membrane that lines the uterus—to grow; and it also elaborates progesterone, or corpus luteum hormone, which causes the endometrial glands to secrete and transforms connective tissue (stroma) into

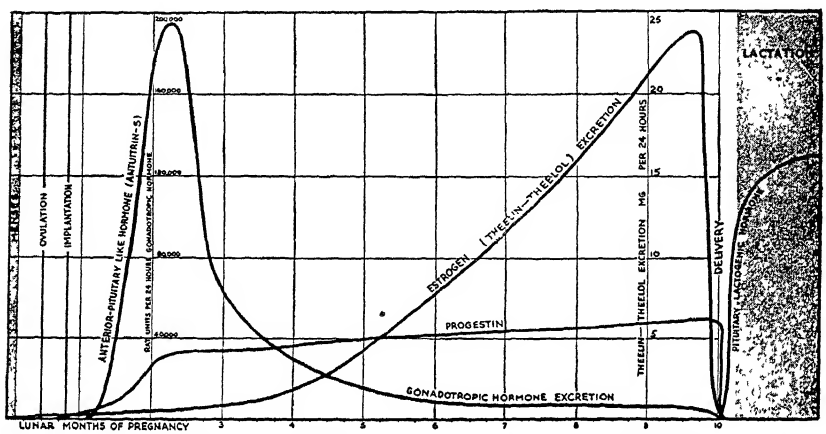


Fig 133.—Hormone excretion in pregnancy. Three important hormones, which profoundly influence physiologic conditions in pregnancy, are excreted by the kidneys. Chorionic gonadotrophin (p 488) appears in the urine; twenty-four-hour excretion rises from less than 20,000 rat units to about 200,000 rat units during the first two lunar months. After a rapid decrease in excretion a level of approximately 10,000 rat units is reached at the sixth lunar month and maintained until delivery.

Combined theelin-theelol excretion in twenty-four hours does not rise beyond 5 mg. during the first half of pregnancy; it increases rapidly during subsequent months, reaching a peak of more than 20 mg during the tenth lunar month Precipitous decline occurs during the last week, values reaching the non-pregnant normal at or shortly after parturition.

Corpus luteum hormone is essential to early pregnancy and plays a vital rôle in the pregravid phase of each menstrual cycle; it is excreted as pregnandiol. After the second lunar month, the level of progestin mounts slowly until parturition, after which it drops sharply. (*Therapeutic Notes*, July, 1938, p. 197, courtesy of Parke, Davis & Co.)

decidua-like cells. These changes are necessary for the implantation of the fertilized ovum.

The normal menstrual cycle occurs only when pregnancy has been averted. Here the hormones involved ultimately fall off in concentration and the endometrium degenerates (Fig. 132).

Where impregnation has occurred, the corpus luteum increases in size and continues to function until nearly the end of pregnancy.

Figure 133 shows the comparative concentration of three of the hormones eliminated (in the urine) during pregnancy. One of them, a gonadotrophic hormone found in pregnancy urine (see p. 488), is used as the basis for the Aschheim-Zondek test for pregnancy. One

method is to inject the urine into immature mice. On the fourth day, the animals are killed and the ovaries examined for hemorrhagic spots (blutpunkte) and yellowish protrusisons (developed corpora lutea).

The Friedman test for pregnancy, a modification of the Aschheim-Zondek test, makes use of the rabbit. The urine under examination is injected into the marginal ear vein of a mature female rabbit. The ovaries are examined twenty-four hour later. The presence of ruptured or hemorrhagic follicles is an indication of pregnancy.

The hormones of the estrin type, of which estrone or theelin is one, are detected by the Allen and Doisy test: the production of estrus (with complete cornification of the vaginal mucosa as judged from a

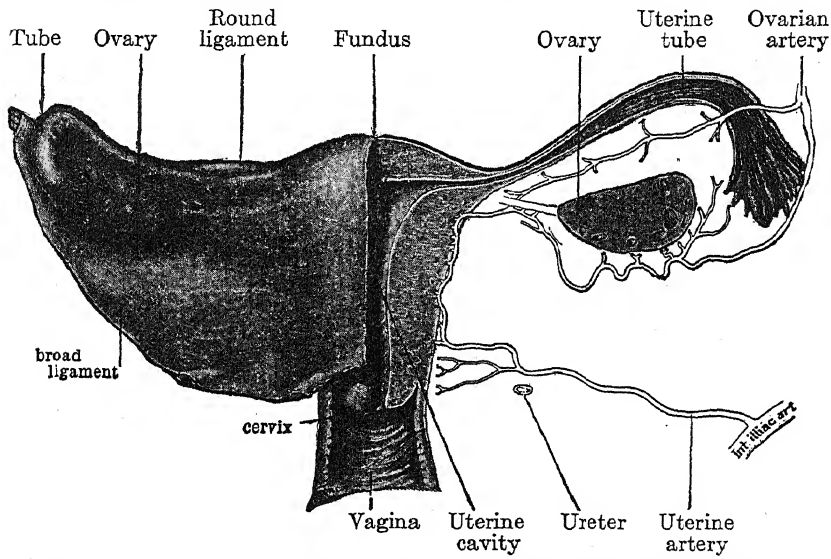


Fig. 134.—Uterus and associated organs. The left half shows posterior view and right half a diagrammatic section. (From Pitzman, *Fundamentals of Human Anatomy*, C. V. Mosby Co.)

smear) in ovariectomized sexually mature rats. The basis of the test for progesterone (progestin), as developed by Corner and Hisaw, is that it exerts a specific proliferative action on the uterine endometrium.

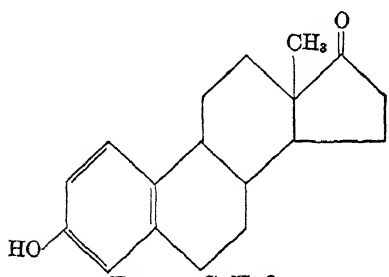
One method of obtaining active extracts from pregnancy urine involves, first, the fact that the active factors are soluble in fat solvents; second, that the hormones can be saponified without destruction; and third, that they can be recovered after saponification by first acidifying (in this case saturating the solution with CO_2) and extracting with ether.

In an alcohol-benzene mixture, most of the estriol (see below) is taken up by alcohol and almost all the estrone by benzene.

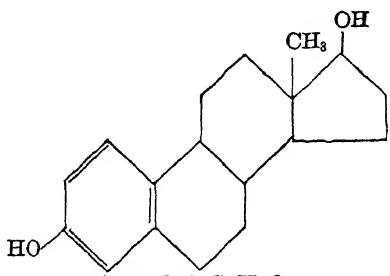
Doisy and Butenandt, in 1929, isolated the follicular hormone (estrone) from pregnancy urine. In 1931, Butenandt obtained a hormone from male urine (androsterone). In 1934, W. Allen, Wintersteiner, Butenandt, and others obtained a crystalline corpus luteum hormone (progesterone) from swine ovaries.

Female Hormones (Estrogens).—So far, five substances of the "estrone" (theelin) group have been isolated: (1) estrone (pregnancy urine, stallion urine, mare urine, and palm kernels); (2) estradiol (ovaries and mare urine); (3) estriol (placenta, pregnancy urine); (4) equilin (mare urine); (5) equilenin (mare urine).

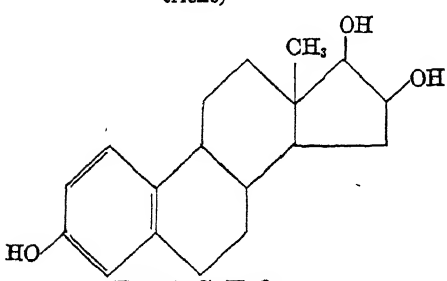
The principal hormone produced by the ovary is estradiol. Estriol is the chief hormone found in human pregnancy urine and in human placenta. (For a drawing of the uterus, etc., see Fig. 134.)



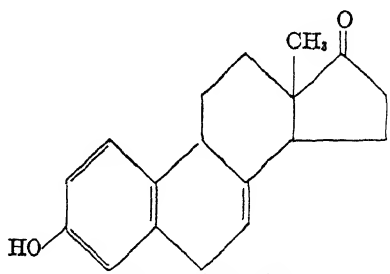
Estrone, $C_{18}H_{22}O_2$.
(Theelin)
(3-Hydroxy-17-keto-Δ1,3,5-estratriene)



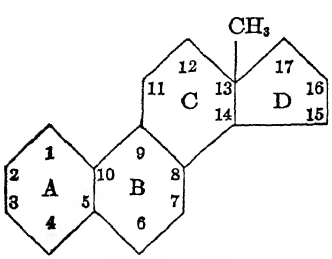
Estradiol, $C_{18}H_{24}O_2$.
(Dihydrotheelin)
(3,17-Dihydroxy-Δ1,3,5-estratriene)



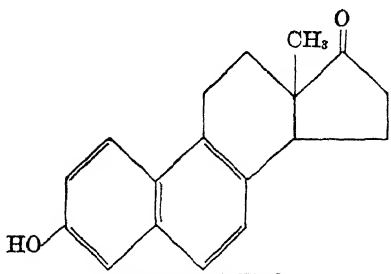
Estriol, $C_{18}H_{24}O_3$.
(Theelol)
(3,16,17-Trihydroxy-Δ1,3,5-estratriene)



Equilin, $C_{18}H_{20}O_2$.
(3-Hydroxy-17-keto-Δ1,3,5,7-estratetraene)

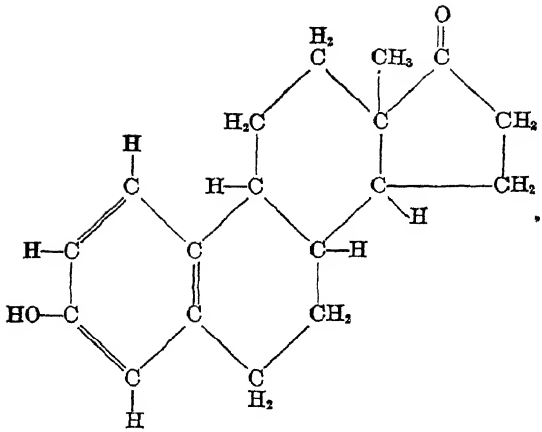


Estrane, $C_{18}H_{30}$.
("Mother substance")

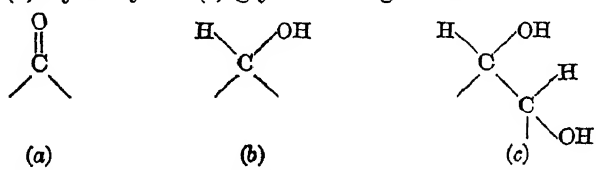


Equilenin, $C_{18}H_{18}O_2$.
(3-Hydroxy-17-keto-Δ1,3,5,6,8-estrapentaene)

Since some of the rings in the compounds are true benzene rings and others are hydrogenated, one of them, estrone, is rewritten again as follows:

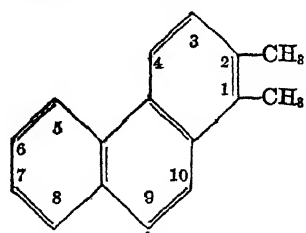


In all of these compounds the first ring is a truly aromatic one, and the OH represents a phenolic hydroxyl group. In equilenin, the second ring is also aromatic. At the other end of the molecule, we find (*a*) carboynl, (*b*) hydroxyl or (*c*) glycol arrangements:



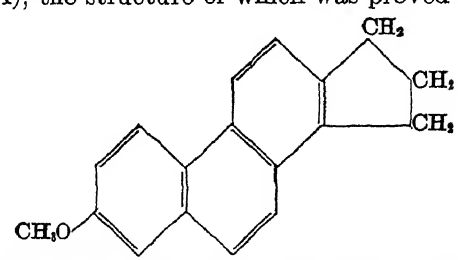
(*a*) (*b*) (*c*)

That these compounds have the four-ring structure given to them was made highly probable by Butenandt when he obtained 1,2-dimethylphenanthrene from estriol by fusion with alkali and reduction with selenium and zinc dust. But further confirmation came from the



1,2-Dimethylphenanthrene.

work of Cook, who converted estrone into a derivative of cyclopentanophenanthrene (A), the structure of which was proved by synthesis:



7-Methoxy-1,2-cyclopentanophenanthrene (A).

(Notice that phenanthrene compounds are numbered differently from compounds derived from estrone.)

This chemical work made it clear that the female hormones belonged to the sterol group, of which cholesterol (p. 39) is so prominent a member This also means that these estrogens are chemically related to androgens (p. 513), to hormones in the adrenal cortex (p. 503), to progesterone (p. 512) and to bile acids (p. 228).

As has been pointed out, estradiol is found mainly in the ovaries, and it is believed to be the mother substance of estrone and estriol.

The metabolic pathway of the estrogens, in so far as they have been studied, suggests the following:

$$\left.\begin{matrix}\text{estradiol}\\ \downarrow\uparrow\\ \text{estrone}\end{matrix}\right\} \rightarrow \text{estriol}$$

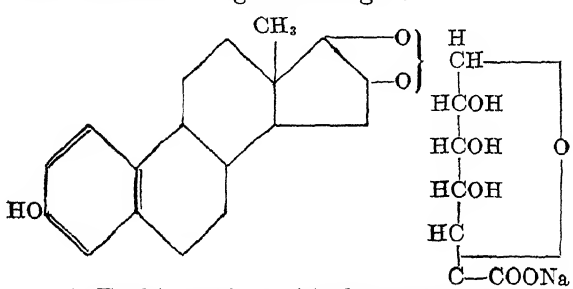
The ovaries and the uterus do not seem essential for these transformations. Men have been fed estrone and estriol has been recovered from the urine.

Suggestive experiments indicate that the liver has the ability to inactivate estrogens. On the other hand, this "inactivation" may be due, in part, to the conversion of the more potent estradiol into the less potent estrone and estriol—a conversion which the liver, apparently, is able to perform.

Neither estrone, estradiol nor estriol have so far been synthesized; but Bachmann has obtained equilenin from a phenanthrene derivative; and since equilenin can be reduced to estradiol, one may speak of the artificial production of estradiol.

Estradiol occurs in two forms, the α-estradiol and the β-estradiol; the difference between the two depending on the space relationship of the OH group at position 17. When estrone is reduced to estradiol, the α-form is the chief product. The α-form is 30 times more potent than the β-variety.

The estrogens are largely excreted in a combined form with glucuronic acid (I). The combined form is more water-soluble and less biologically active than the original estrogen.



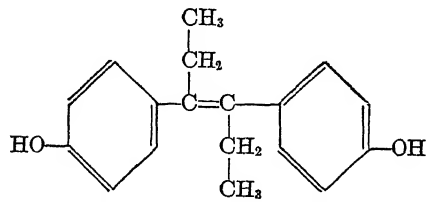
1. Estriol combined with glucuronic acid.

There is evidence that the estrogens—or at least estrone—may be partly eliminated in combination with sulfuric acid.

As in most conjugations of this kind, the reaction probably occurs in the liver. (See under detoxication, p. 246.)

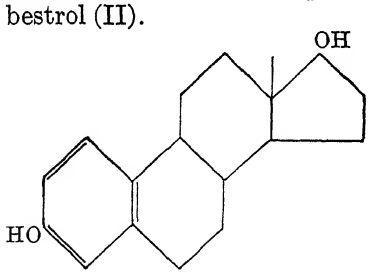
Continuous injections of estrogens may damage the reproductive organs. This applies both to the male and the female. These results are due to a depressant effect on the pituitary.

Diethylstilbestrol.—A synthetic compound, 4,4'-dihydroxy-$\alpha\beta$-diethylstilbene (A), commonly known as *diethylstilbestrol,* is two to three times as potent (when injected into ovariectomized rats) as estrone, under similar conditions (Dodds).

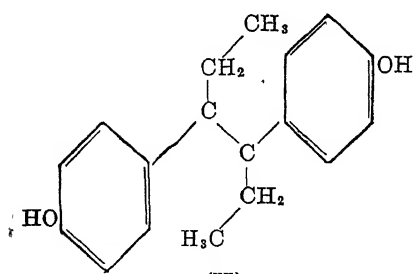


The advantages of this synthetic substance is that, aside from its greater potency as compared to estrone, it can be given orally, whereas the estrogens have to be injected; and—a by no means unimportant factor—the stilbestrol is a comparatively cheap substance. There is some evidence that, under certain conditions, this synthetic product may give rise to toxic symptoms.

Written spatially as shown, Dodds and coworkers believe that there is some relationship between estradiol (I) and the diethylstilbestrol (II).



(I)

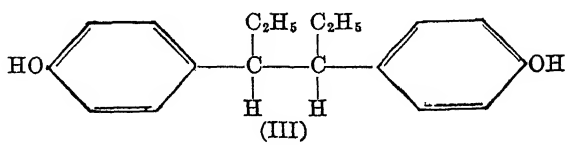


(II)

In fact, one hypothesis is that the stilbestrol compound is actually converted in the body into a true estrogen.

On the other hand, it is equally possible that a compound need not necessarily have a sterol configuration before it induces estrogenic changes in the body.

A compound similar to diethylstilbestrol, dihydrodiethylstilbestrol (III) (also known as "hexostrol") is about as potent as estradiol.



(III)

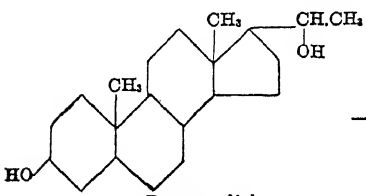
After having tried many compounds, the conclusion seems warranted that the 4-hydroxyphenyl group plays an important rôle in the physical activity of the product.

Diethylstilbestrol is excreted, to some extent at least, as the glucuronide.

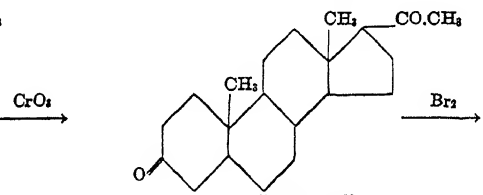
When used clinically, the acetate or propionate is applied because the absorption is slower.

Corpus Luteum Hormone.—This hormone is found in the female during the second stage of the monthly cycle. It makes its appearance in the cavity of the ruptured follicle after the egg has developed and continues the action of the female hormone in the development of the mucous membrane of the uterus. It acts on the uterus so that this organ may receive and nourish the fertilized ovum. "When an ovum begins its journey through the fallopian tube, the follicle from which it took origin gives place to the corpus luteum, and this organ thereupon delivers into the blood stream a substance, progesterone, that has the property of causing extensive development of the endometrium, preparing the uterus for the reception and nutrition of the embryo." (Corner.)

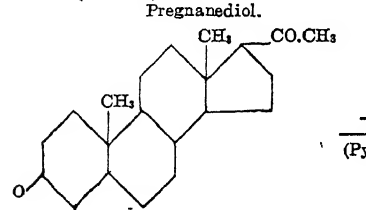
Isolation.—The hormone known as *progesterone* (progestin) has been prepared in crystalline form from ovarian extracts by a number of workers (Butenandt, W. Allen, Wintersteiner, Slotta, etc.). Its formula is $C_{21}H_{30}O_2$ and it is a tetracyclic diketone. It has been prepared artificially from two substances: from pregnanediol (Marrian) found in pregnant urine; and from stigmasterol, a plant sterol.



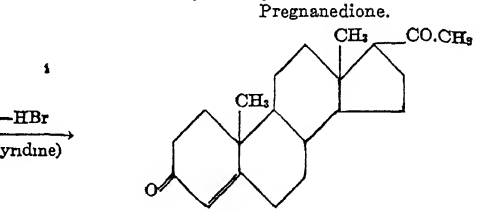
Pregnanediol.



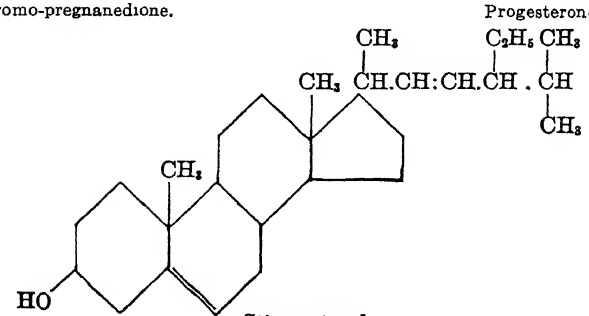
Pregnanedione.



4-Bromo-pregnanedione.



Progesterone.



Stigmasterol.

Metabolism.—Pregnanediol is the chief product of excretion of the corpus luteum hormone. During the latter half of the menstrual cycle, from 1 to 10 mg. daily of sodium pregnanediol glucuronidate may be recovered from the urine. The presence of pregnanediol in the urine indicates a progestational endometrium, and its absence a follicular endometrium.

An important contribution to the metabolism of progesterone has been made by Bloch. Cholesterol, containing deuterium in the side chain and in the nucleus, was fed to a woman in the eighth month of pregnancy. At this stage enough pregnanediol glucuronidate was excreted in a day for a deuterium analysis. The glucuronidate was isolated and found to contain significant concentrations of the isotope.

Since pregnanediol, in this instance, is a metabolic product of progesterone, this experiment implies that cholesterol can be transformed in the body into progesterone.

The same author had already shown that bile acids can be formed from cholesterol (p. 228).

Cholesterol, then, may perhaps be regarded as a precursor of steroid hormones and bile acids, though the quantities of cholesterol used for such conversions "are negligible as compared to those present in animal tissues."

These metabolic activities of cholesterol apply to tissues other than the brain, for though relatively large quantities of the sterol are found in the brain, such brain cholesterol has been shown to be metabolically inert: "it is not regenerated at a detectable rate."

The female secretes not only estrogens (female hormones) and progesterone, but a considerable amount of androgens (male hormones). Androgens are probably formed by the adrenal cortex rather than by the ovaries.

Male Hormones (Androgens).—"The testicle," writes Moore, "exercises two principal functions; it produces spermatozoa, which are necessary for fertilization, and secretes a substance or substances (hormone) that plays an important rôle in the organism. This hormone places in function numerous special accessory reproductive organs: epididymis, vas, prostate, seminal vesicles, penis, etc., that make possible the delivery of spermatozoa to the place where fertilization can occur; and at least in sub-primate vertebrates the hormone initiates the sexual drive, or inclinations to mate with females. The sex urge in man is not so clearly or exclusively dependent upon hormone action since imitation, custom, and psychology play such a great rôle in human conduct." (See Fig. 135.)

A number of substances respond to the male hormone tests (comb growth in the capon, for example). An active extract, responding to the comb test, may be prepared from the urine of males by extracting an acidified portion with an organic solvent, such as chloroform, evaporating the chloroform from the extract, heating the residue with

sodium hydroxide, extracting the active material with ether; evaporating the ether, and incorporating the residue with oil (Funk, Harrow, and Lejwa).

A potent extract injected into a castrated cock (Fig. 136) over a period of eighteen days will produce the result (comb and wattles) shown in Fig. 137.

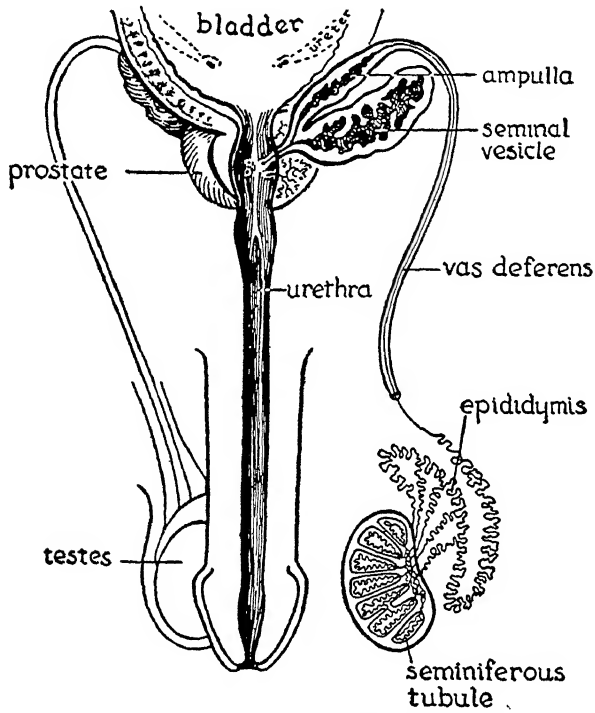
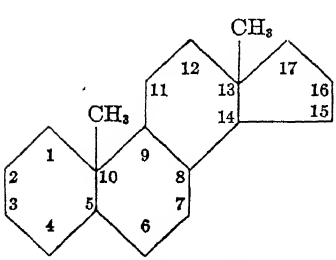
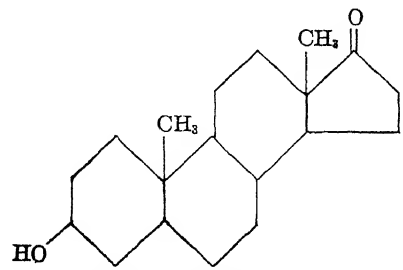


Fig. 135.—The male reproductive system. (Drawn by E. M.) (Gerard, *The Body Functions*, John Wiley and Sons, Publishers.)

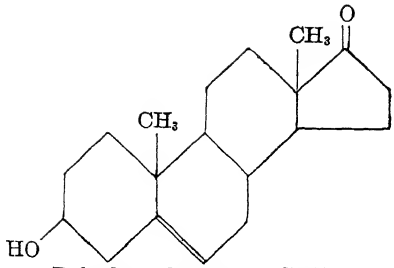
Among the naturally occurring compounds belonging to the male hormone group are androsterone, $C_{19}H_{30}O_2$, in male urine, dehydroandrosterone, $C_{19}H_{28}O_2$, in testes; androstanedione, $C_{19}H_{28}O_2$, in testes; and testosterone, $C_{19}H_{28}O_2$, in testes.



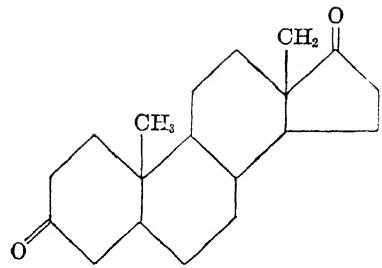
Androstane, $C_{19}H_{32}$.
("Mother substance")



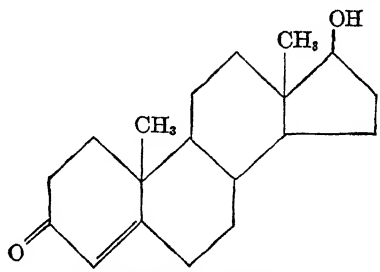
Androsterone, $C_{19}H_{30}O_2$.
(3-Cis-hydroxy-17-keto-androstane)



Dehydroandrosterone, $C_{19}H_{28}O_2$.
(3-Trans-hydroxy-17-keto-Δ^5-androstene)



Androstanedione, $C_{19}H_{28}O_2$.
(3, 17-Diketoandrostane)

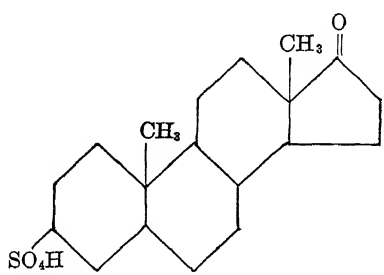


Testosterone, $C_{19}H_{28}O_2$.
(3-Keto-17-hydroxy-Δ^4-androstene)

Of these substances, testosterone is physiologically the most powerful.

Elimination.—There is evidence for the belief that the testosterone elaborated by the testes (see Fig. 135, p. 514) is converted to androsterone before the latter is eliminated in the urine.

The estrogens, it has been seen, are eliminated, at least partly, as glucuronides (estriol; pregnanediol) and possibly as sulfate (estrone). The androgens, so far, have been isolated in the urine as sulfate derivatives (androsterone; dehydroisoandrosterone). The androsterone sulfate has the formula



Androsterone and dehydroandrosterone, the first strongly androgenic and the second but mildly so, are found in both male and female urines. Since these substances have been isolated from the urine of eunuchs and from the urine of ovariectomized women, the genital glands are not always necessarily the originators of such compounds. There is reason to believe that under certain conditions the adrenal glands manufacture these substances (see p. 503).

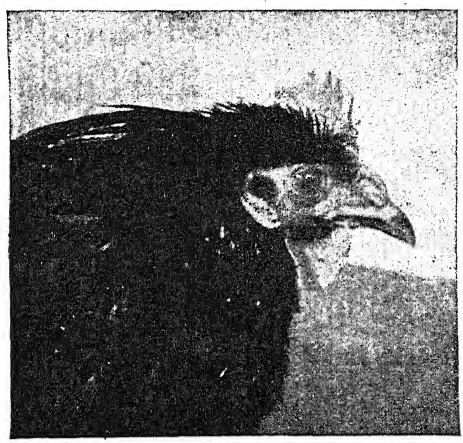


Fig. 136.

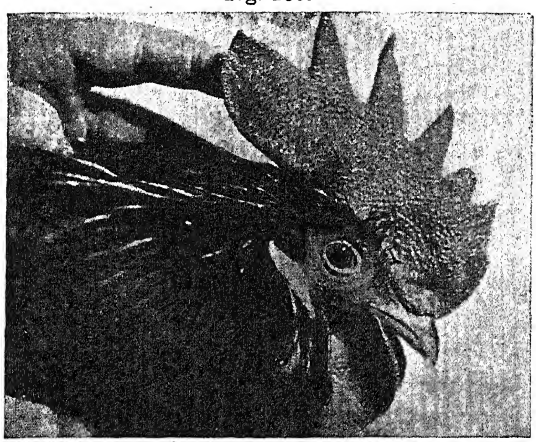
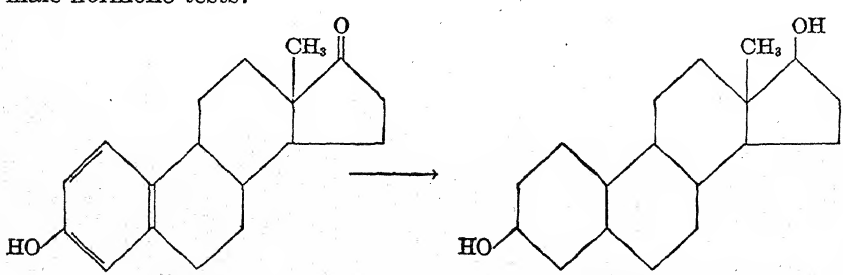


Fig. 137.

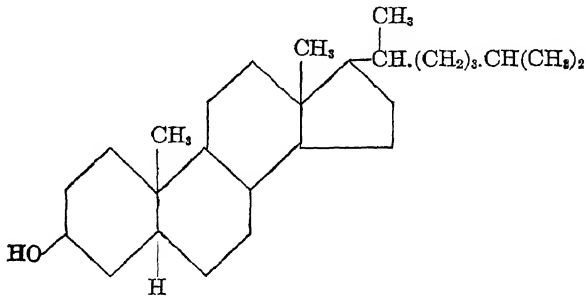
Figs. 136, 137.—(Koch, *Harvey Lectures*, Ser. 33, p. 205, Williams and Wilkins Co., Publishers.)

Chemistry.—Based on somewhat analogous chemical work with the female hormones, it was possible for Butenandt to predict the tetracyclic nature of these substances. This view was further strengthened by the successful reduction of estrone into octahydroestrone (absorption of 8 hydrogen atoms), giving a product which responded to male hormone tests:



Estrone. Octahydroestrone.

A very important advance in our knowledge of these structures was the success attained by Ruzicka in converting a cholesterol derivative into androsterone, one of the male hormones. Specifically, dihydrocholesterol, as the acetate, is treated with chromic oxide, and androsterone can be recovered from the oxidation products.*

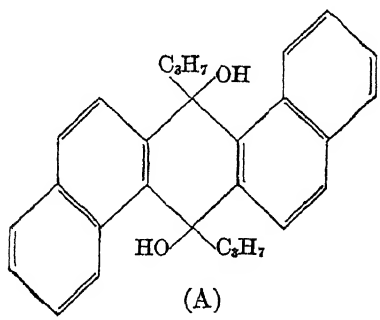


Dihydrocholesterol

Testosterone has also been prepared artificially, starting with dehydroandrosterone.†

Figure 138 (p. 520) presents some endocrine interrelationships.

Synthetic Substances Showing Physiological Properties (Refer also to p. 511).—Slight modifications in structure—the change of a carbonyl group to a secondary alcoholic group—may markedly change the physiological activity of the compound. Many compounds have been made which can produce estrus; some are but distantly related to the original estrane structure. For example, a derivative of dibenzanthracene (A) corresponds in its activity to the natural estriol.

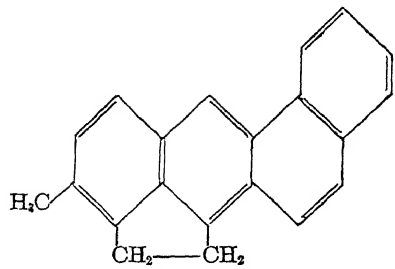


(A)

This substance (A), incidentally, shows not only estrogenic properties but also "cancer-producing" activities; for, when applied continuously to a mouse over a period of time, there develops a malignant tumor. The same is true of a compound, methylcholanthrene, which may be obtained artificially from desoxycholic acid, one of the acids of the bile (Fieser).

* Ruzicka's synthesis is given in the appendix, p 572.

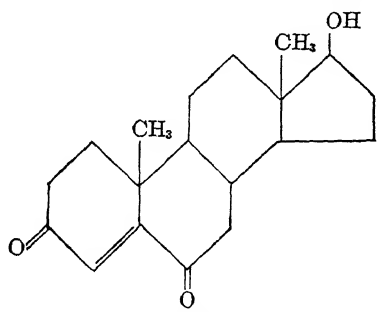
† Ruzicka's method, as summarized by Koch, will be found in the appendix, p. 573.



Methylcholanthrene.

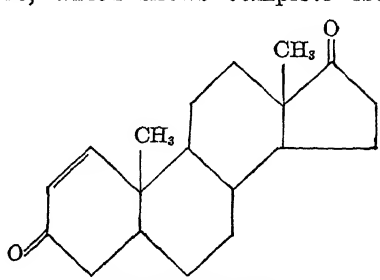
We have already discussed stilbestrol, a synthetic compound with powerful estrogenic properties (p. 511).

Butenandt and his coworkers (Riegel, Dannenberg) have also interested themselves in modifying structures to bring about a reversal in physiological action. For example, 6-oxo-testosterone, derived from



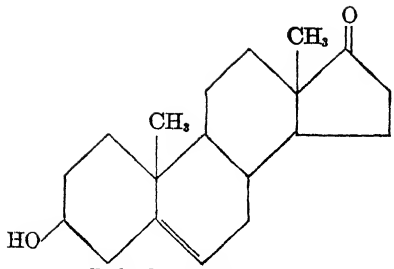
6-Oxo-testosterone.

testosterone, shows no testicular action, but a very definite follicular action. This change from "male hormone" to "female hormone" activity is also illustrated by the conversion of androstanedione to $\Delta^{1,2}$-androstenedione, which shows complete estrus in a castrated

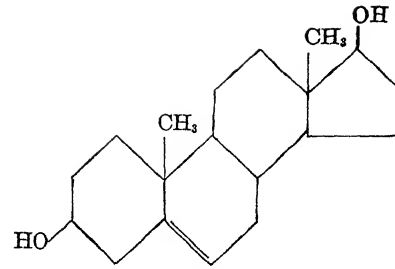


$\Delta^{1,2}$-Androstenedione.

female mouse when injected in $4 \times 500\gamma$ amounts; but even 4 mg. of the material still fails to show growth in the comb of the capon. "In this example," as Butenandt says, "the difference between a male and female substance is merely the position in the double bond in ring I." But he has even gone one step further. He has obtained a substance, androstenediol, from dehydroandrosterone, which shows both "male" and "female" properties.



Dehydroandrosterone.
♂



Androstenediol.
♂ ♀

Production of Cancer by Chemical Compounds.—It has already been pointed out that a derivative of dibenzanthracene (p. 517) and methylcholanthrene (p. 518) exhibit "cancer-producing" activities. It has also been observed that in adrenal cortical tumor, resulting in adrenal virilism, large amounts of male hormones are excreted.

In one case, Fieser points out, the amount of hydroandrosterone excreted was 100 times as much as in the normal. Fieser is of the opinion that in abnormal metabolism—such as the example just cited—a sterol compound (represented by one of the sex compounds) may, perhaps, be transformed into a "carcinogen," or cancer-producing product, of the cholanthrene (p. 518) type.

"An abnormal process leading to the formation of a carcinogen may be only slightly differentiated from normal sex hormone metabolism. Suspicion as to the point of origin of a "degenerated biocatalyst" centers around the adrenal cortex partly because steroids of the adrenal cortical type appear to be the likely precursors of all the sex hormones and partly becasue this gland, particularly when hyperactive, appears to maintain conditions favorable for dehydrogenative processes."

This subject of cancer produced by chemical compounds has an interesting history. In 1775 Pott described scrotal cancer in chimney sweeps developed by contact of chimney soot with the abraded skin. Later, with the development of the coal tar industry, various clinical reports appeared pointing to tar products as causative agents of cancer in man.

In 1912 Yamagiwa and Ichikawa produced cancer in the rabbit's ear by continuous application of gas-works tar. Several compounds were later isolated from coal tar by Cook which induced this malignant disease. In general, these substances were, chemically, derivatives of condensed benzene rings.

Working in an entirely unrelated field, Leo Loeb in 1916 showed that the implantation of ovarian tissue into castrated male mice resulted not in female breast structure but in cancer of the breasts. Later, with the isolation of the female hormones, it developed that estrone, estriol and estradiol (p. 508) are able to produce breast cancer in mice. Structurally, these compounds were not unrelated to the carcinogenic compounds isolated by Cook from coal tar.

An unexplained fact is that different strains of mice react quite

differently towards these carcinogenic substances: some strains are immune to breast cancer otherwise developed by the injection of female hormones. Also, different species show decided variations. Under

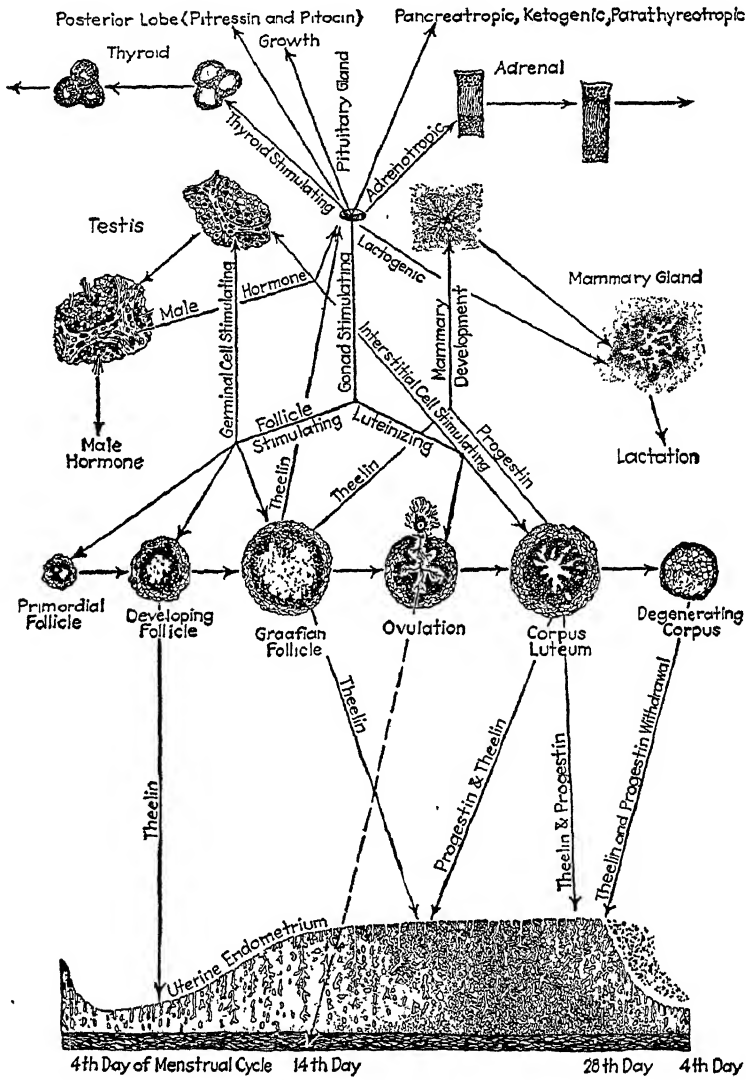
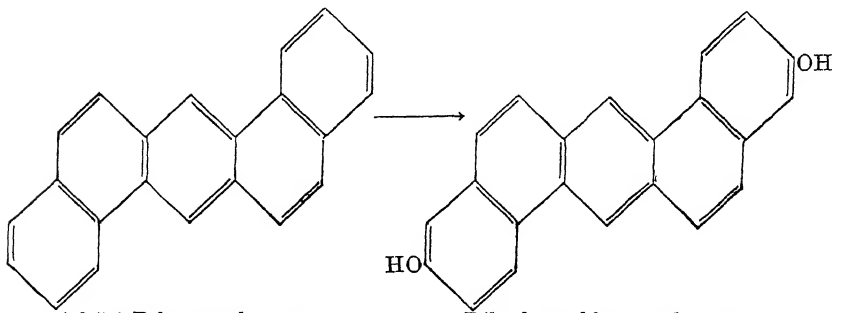


Fig. 138.—Enodocrine interrelations. (Schmidt and Allen, *Fundamentals of Biochemistry*, McGraw-Hill Book Co.)

the conditions of the experiments, guinea-pigs are less likely to develop cancer than mice, and rabbits even less so than guinea-pigs; and it is not at all certain that in primates, in monkey and in man, either the female hormones or the organic products obtained from tar are able to produce cancer.

All this has led Rhoads to the theory that possibly "various species and various strains of the same species may differ in the manner in which they handle, destroy or detoxify those substances which have been shown to produce cancer."

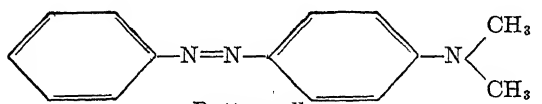
In connection with this view, it had been observed that one of the outstanding cancer-producing substances, dibenzanthracene was excreted as the dihydroxy analogue. The dihydroxy derivative, in con-



1,2,5,6-Dibenzanthracene. Dihydroxydibenzanthracene.

tradistinction to its mother substance, showed no cancer-producing properties; from which it was concluded that in the attempt to protect itself, the rabbit—the animal experimented with in this instance—detoxified the dibenzanthracene. Since detoxication is primarily a function of the liver, it was assumed that such detoxication had occurred in this organ.

An experiment by Kinosita suggested further possibilities. He observed that the dye, butter yellow, caused liver cancer in rats fed a diet of unpolished rice and carrots. Such cancer was prevented by the addition of yeast or liver to the diet. Since both yeast and liver are



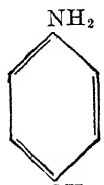
Butter yellow.

rich in the vitamin B complex, the possibility of some relationship to vitamins could not be overlooked. That some such relationship does exist was shown by the addition of riboflavin and casein to the diet of unpolished rice and carrots, resulting in distinct protection. The carrots and the unpolished rice lacked riboflavin and some, as yet, unidentified constituent in casein.

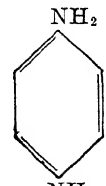
How did the riboflavin, for example, protect the animal? By combining with the cancer-producing chemical and detoxifying it? By developing its corresponding enzymes, the yellow oxidizing enzymes?

In this connection, experiments involving coenzyme I (p. 87), containing the nicotinic acid amide (p. 392), are of some point. The livers of rats fed butter yellow showed a marked decrease of coenzyme I. When such animals were fed yeast, not only were they protected from cancer, but the amount of the coenzyme in the liver was normal.

Butter yellow itself is decomposed in the body into several products. Both free and actylated forms of *p*-aminophenol and *p*-phenylenediamine have been recovered from the urine.

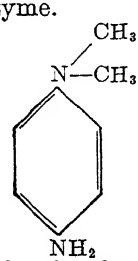


p-Aminophenol



p-Phenylenediamine.

The effects of these various compounds on the activity of the coenzyme system *in vitro* was tried. *p*-Phenylenediamine was the only one which inhibited the activity of the enzyme. Since the dimethyl derivative, though not isolated, was probably a precursor of the *p*-phenylenediamine, the compound was also tried and found to be even more toxic to the coenzyme.

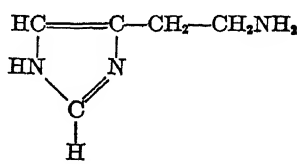


Dimethyl-*p*-phenylenediamine.

"The administration of at least one carcinogenic chemical," writes Rhoads, "injured normal cells by interfering in some way with an enzyme system which is essential for their normal chemical function and so their normal life."

GASTRO-INTESTINAL HORMONES*

In so far as the secretion of saliva is concerned, a hormone mechanism seems to play no part. A humoral mechanism may, in part, be involved in gastric secretion. The injection of an acid extract of the pyloric mucosa stimulates secretion. This stimulation was said to be due to a hormone, to which the name *gastrin* had been given. Ivy has isolated histamine from this extract and finds it the only secretory excitant present.



Histamine.

However, this view has been disputed. Komarov, for example, claims to have obtained histamine-free extracts from the pyloric

* Refer to Chap. 10.

mucosa which contain two active materials: one of these stimulates the gastric glands (gastrin), and the other, the external secretion of the pancreas (similar to secretin).

Enterogastrone, a substance which inhibits gastric secretion, has been obtained in concentrated form from the upper intestinal mucosa. A probable excretory product, and showing some of the properties of the mother substance, is *urogastrone*.

The mucosa of the upper part of the intestine is responsible for two hormones, *secretin* (p. 224) and *cholecystokinin*. Bayliss and Starling obtained an acid extract of the mucosa, which, when injected, caused a flow of pancreatic juice. The active material in this acid extract was named "secretin," and substances of the type represented by secretin were called "hormones" (from the Greek, "to excite").

Crystalline products of secretin have been obtained. Whether the substance is a protein or is of smaller molecular dimensions (polypeptide) is not clear.

Extracts from the mucosa of the upper part of the intestine (freed from histamine and choline) give, when injected, a prolonged contraction of the gallbladder with evacuation. The substance which stimulates this contraction of the gallbladder is called "cholecystokinin" (Ivy) which, chemically, resembles secretin, though it is not identical with it.

Suggestions have been made that there may be several additional hormones in the intestine: a substance which stimulates the production of insulin (incretin);* and a substance which augments the motility of the intestine.

PLANT HORMONES†

While plant hormones belong, more specifically, to plant biochemistry and not to animal biochemistry, plant and animal hormones may be related. At any rate, plant hormones have been obtained from the urine of human beings and a "female hormone" has been obtained from plant extracts.

In one series of experiments, conducted by Kögl, a substance was isolated in a chemically pure state which affects the curvature (cell stretching) in plants. The "growth substance" to be tested— which, for example, is found in the tips of the coleoptile of oats—is placed in contact with cubes of agar-agar, which allow the active material to diffuse into it. If such cubes are placed on the cut area of the coleoptile, curvature results; and the extent of such curvature is proportional to the concentration of active material produced (Fig. 139).

While the tips of oats and corn, fungi and yeast contain the hormone, Kögl found urine (male and female) to be the most convenient source material. He isolated an active acid, $C_{18}H_{32}O_5$, which formed

* See "Insulinotropic hormone," p. 491.

† One finds various names in the literature: Auxins; growth hormones; growth regulators; phytohormones; growth substances. These include true hormones, like the auxins, which are found in the plant, and many substances which affect growth (positively and negatively), but which are not necessarily part of the plant structure.

a lactone and contained three hydroxyl groups. To this was given the name "auxin A." From malt and from maize germ, another acid,

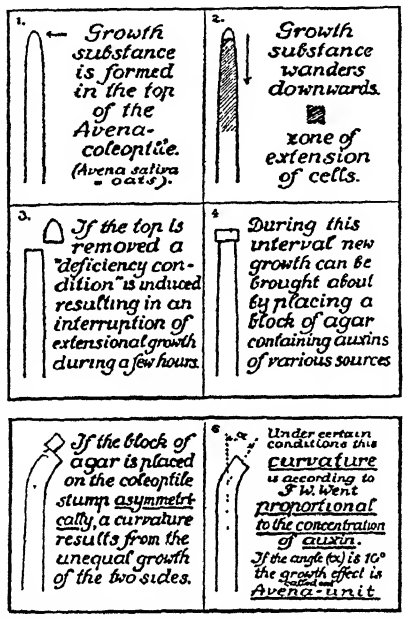
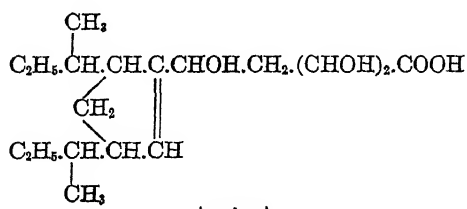
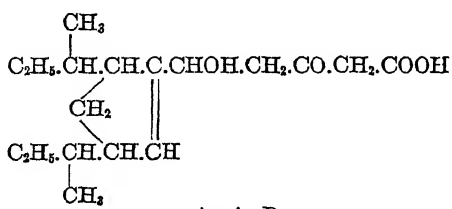


Fig. 139.—Estimation of plant hormone. [Kögl, *Chemistry and Industry*, **57**, 49 (1938.)]

$C_{18}H_{30}O_4$, isomeric with the lactone from auxin A, was obtained; this substance was called "auxin B." Exhaustive and beautiful chem-



Auxin A.



Auxin B.

ical investigations by Kögl, recalling the work of Butenandt on the sex hormones, led to structural formulas for these two substances.

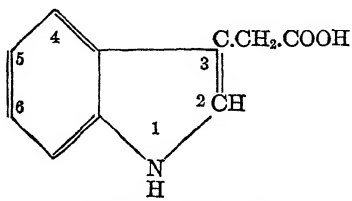
Auxin B is the β-keto acid of auxin A.

Commoner and Thimann have shown that a close connection exists between growth and respiration. Using the *Avena* coleoptile for their

experiments, they have shown that auxin provides a link between growth and respiration. The link is the four-carbon acid respiratory system: malate, fumarate, etc. (p. 388).

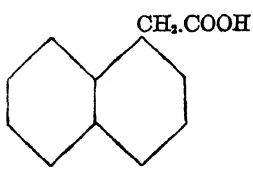
The growth of *Avena* coleoptile sections in sucrose solutions is retarded by substances which inhibit dehydrogenase action; for example, iodoacetic acid. This retardation is overcome by malate and fumarate. Respiration is considerably increased if auxin is added to sections of coleoptile soaked in malate and fumarate.

Substances Showing Growth-Promoting Properties.—Many substances possess this growth-promoting activity. Kögl isolated from

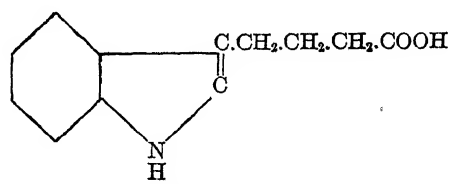


Indole-3-acetic acid.

urine a third substance, indole-3-acetic acid, which exhibited marked activity. Curiously enough, this substance is a bacterial decomposition product of proteins derived, more specifically, from tryptophan. Thimann has shown that the growth-promoting substance in mold cultures is this indole derivative. Hitchcock and Zimmerman have tried many substances for their growth-promoting properties and find them very widely distributed—from such a simple substance as ethylene to indole, benzene, naphthalene, and even anthracene derivatives. They found the most effective root-forming substances to be α-naphthalene acetic acid and indole-3-butyric acid (Fig. 140),



α-Naphthalene acetic acid.



Indole-3-butyric acid.

although they were not as effective as indole-3-acetic acid for epinastic response of leaves.

Ways in Which Growth Is Affected.—Many substances affect growth in one of a number of ways: anesthesia; prolongation or shortening of dormancy; promotion of cell growth: excessive upward growth (hyponasty), or abnormal downward growth (epinasty); stimulation of root formation, normal, and abnormal; asexual fruiting produced by artificial stimulation and not natural pollination.

All root systems require vitamin B_1 which the plants normally manufacture. If traces of vitamin B_1 (thiamine) are added to tips of

roots kept in a nutrient solution containing mineral salts, sucrose and nitrate, flax roots can be grown at a rate of one inch per day.

Roots of pea and radish require nicotinic acid in addition; and tomatoes do exceptionally well if vitamin B_6 is added. Tomato leaves in ethylene vapor (a few parts per million) grow downwards instead of upwards. This reaction is so sensitive that it can be used as a test for the gas.

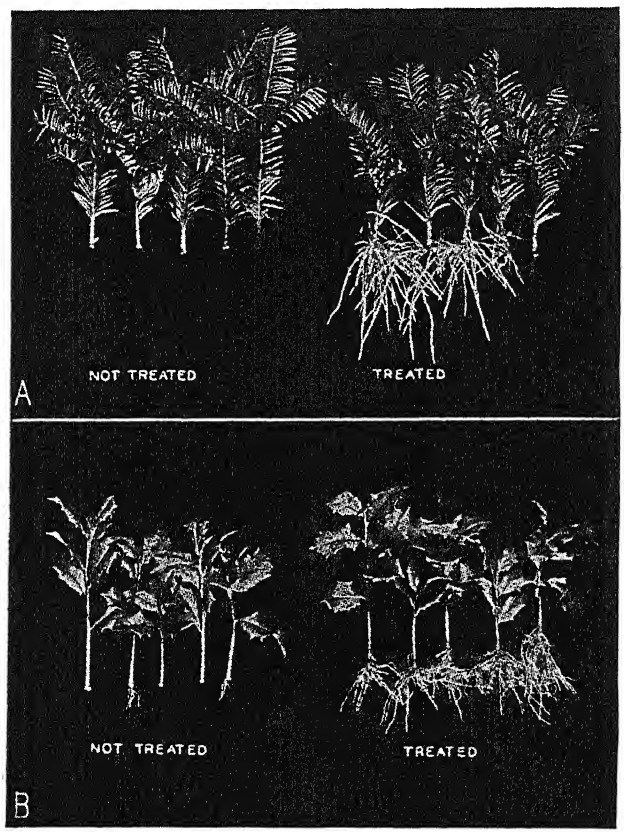


Fig. 140.—Cuttings treated with water solution of indolebutyric acid for twenty-four hours, then planted in the rooting medium. A, Taxus. B, Holly. (Zimmerman, *Ohio J. Science*, **37**, 333.)

Ethylene also has an anesthetic reaction on plants somewhat similar to what it has on animals. The plants in its presence become less sensitive to stimuli. But together with this anesthestic reaction on plants, ethylene may also have a stimulating action on certain parts of the organism. A practical example of this is the coloring of oranges by the gas.

Haberlandt has shown that many plant tissue extracts induce renewed growth in mature plant cells. These extracts can be obtained from ground or heated tissues (for example, from the pods of beans),

and are capable of inducing cell division and cell enlargement in unwounded cells.

The active principles have been called "wound hormones." One active substance has been isolated and proves to be a dicarboxylic acid of the type $HOOC(CH_2)_nCH{=}CH{-}COOH$.

ANTIHORMONES

Collip has shown that repeated injections of various hormones (mainly those belonging to the pituitary group) into animals make them less and less "sensitive" to such hormones. The sera of such animals immunize another animal to the specific hormone under investigation. Collip propounds two possibilities: either that the hormone extract is an antigen and the inhibitory substance in the blood stream is an antibody; or else that the inhibitory substance is a definite substance of an "antihormone" kind.

Marrian and Butler, in a summary of the situation, believe the problem still unsettled. In favor of Collip's theory is that rats develop immunity to the gonadotropic effect of rat pituitary implants; and also that an immune serum may be developed in sheep by the injection of sheep pituitary gonadotropic preparations. On the other hand, in favor of the "antigen" theory is the fact that the power of beef pituitary thyrotropic extracts to induce antihormone formation in the guinea-pig depends upon the method of preparation. Furthermore, inactivated human pregnancy-urine gonadotropic preparations are as effective in inducing antihormone formation as active preparations.

INTERNATIONAL UNITS

Table 82 gives some of the hormone standards in International Units.

TABLE 82.—INTERNATIONAL STANDARDS. (League of Nations Quarterly Bulletin of the Health Organization. Biological Standardization II. November, 1936.)

Substance.	*Nature of preparation.*	*International unit (in mg.).*
Insulin	Dry insulin hydrochloride (1925)	0.125
	Same (1935)	0.045
Estrus-producing hormones	Hydroxy-ketonic form (1932)	0.0001
	Benzoate of the dihydroxy form (1935)	0.0001
Male hormone	Pure crystalline androsterone (1935)	0.1
Corpus luteum hormone	Pure crystalline progesterone (1935)	1.0

Grollman, in his *Essentials of Endocrinology*, summarizes several additional hormone standards:

Adrenal Cortex.—The standardization of desoxycorticosterone acetate is by actual weight in mg. per cc.

Adrenal Medulla.—Epinephrine (adrenaline) is standardized in grams per 100 cc. of solution.

Parathyroid.—One U. S. P. unit is $\frac{1}{100}$ of that amount required to raise the calcium level of 100 cc. of the blood serum of normal dogs 1 mg. within sixteen to eighteen hours. If dihydrotachysterol is used, the standard is the actual weight in mg. per cc.

Pituitary, Anterior Lobe.—For prolactin, the international unit (I. U.) is the activity of 0.1 mg. of the standard assayed by the pigeon crop response. For thyrotropic activity, the unit is the activity of 0.25 mg. of the standard.

Pituitary, Posterior Lobe.—The I. U., applying to pressor, oxytocic and antidiuretic activity, is the activity of 0.5 mg. of standard powdered posterior pituitary.

Thyroid.—Thyroxine is standardized by actual weight in mg. per tablet or vial.

Ovary.—The I. U. has been declared to be 0.1 microgram of α-estradiol benzoate. (Unfortunately, it is difficult to compare the activities of estrone and estradiol; "their relative activities and period of action are not the same.")

Placenta.—The I. U. for the human chorionic gonadotropin is the specific gonadotropic activity of 0.1 mg. of a standardized powder. If equine gonadotropin (from pregnant mare's series) is used, the I. U. is 0.25 mg. of a standard powder.

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CHAPTER 25

THE CHEMISTRY OF THE NERVOUS SYSTEM

A DISCUSSION of the biochemical aspects of the nervous system is reserved for the last chapter of the book, not because the subject is unimportant, but because so little is known about it. Some day, with more facts at our disposal, this chapter will prove to be the most illuminating of all.

Composition.—The gray matter of the brain may contain as much as 80 per cent—and even more—of water. Of the solids present in nervous tissues, some 50 per cent may be due to proteins, of which collagen and neurokeratin are the most abundant, according to Block. Neurokeratin itself, though possibly different from the keratin found in epidermal tissue, is similar to the latter in so far as its general insolubility and its resistance to peptic and tryptic digestion are concerned. Block believes that neurokeratin is possibly the protein in the neurofibrils, the filaments in the nerve cells and their axons.

Relatively small quantities of alkaline phosphates, phosphocreatine, adensinetriphosphate, hexosephosphate and chlorides, carbohydrates, "extractives" (creatine, etc.) and inositol are also present. But aside from the proteins, the materials present in largest quantity (in nerve fibers, at least), and in many ways the most characteristic materials, are the lipids.

The brain of a rat embryo contains 10 per cent of fatty acids; so does the liver. When thirty days old, the fatty acids of the liver still remain 10 per cent, but those of the brain have increased to 20 per cent.

The "turnover" of these lipids in the brain is a slow process by comparison with other tissues of the body. Using deuterium as the "tracer," it can be shown that in the adult brain, some 20 per cent of the fatty acids are replaced in a week. In the liver, on the other hand, the "turnover" is as high as 50 per cent in one day.

The chemistry of these lipids has already been discussed (Chap. 3). These substances include lecithin, cephalin, sphingomyelin, cerebrosides and sterols (particularly cholesterol) besides the true fats. While not peculiar to nervous tissue, some, like the cerebrosides, are rarely found in any other part of the body; and they are certainly present in abundance.

A disease in childhood, known as the *Nieman-Pick disease,* is characterized by an increase in the sphingomyelin of the brain.

The "cephalin" in brain is not a definite compound, as was supposed, but apparently a mixture of several compounds.

Folch has shown that 40 to 70 per cent of the nitrogenous constituent is not ethanolamine (p. 37), but *l*(+)serine (p. 51).

From the so-called "cephalin" a phosphatide was separated which had all of its nitrogen as serine; this compound was given the name, "phosphatidyl serine."

Still another phosphatide present in this "cephalin" contains inositol (p. 147).

The Metabolism of Nerves.—The unit of the nervous system, the neuron, consists of the cell, the dendrites and the axon (Fig. 141). The axon (or axis-cylinder) is the central core of a nerve fiber. The nervous impulse is propagated along the nerve with a velocity of 27 meters per second in the frog, and more rapidly in the mammal. There is a change in electric potential: the portion of the nerve in action is electrically negative, as compared to "resting" portions, in front and

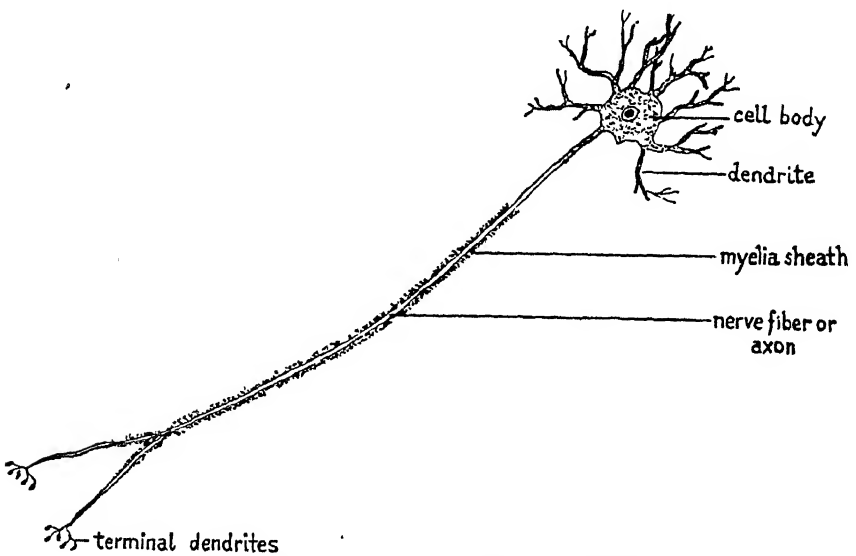


Fig. 141.—A nerve cell or neuron. (Dawson.)

behind. The fatigue of the nerve comes only after relatively long periods of activity.

It is possible, particularly when working with cold-blooded animals, to excise a nerve and keep it "in action" for some time, assuming a temperature which is low enough, and providing, also, that the moisture is suitable. Under such conditions, it is possible, by the use of instruments delicate enough, to measure the production of heat generated during the course of the activity of the nerve and to examine a number of electrical properties.

The fact that heat is produced during the conduction of an impulse is evidence of a metabolic process. Such a condition is apparently not merely a physical, but also a chemical phenomenon. Although the amount of heat produced is small enough, it has been measured; it amounts to 141×10^{-5} calories per gram of nerve for a stimulus lasting ten seconds. The heat is evolved in two stages; a small amount of heat —from 2 to 3 per cent of the total—is produced; and the remainder is

evolved over a period which may last half an hour or so after the stimulation.

Oxygen is absorbed by the nerve at rest; but during and following stimulation much more is absorbed. The Q_{O_2}* of resting frog nerve at 15° is −0.08, and of stimulated frog's nerve, −0.3 The nerve, it is known, will conduct for a comparatively long time in an atmosphere of nitrogen. Upon the admission of oxygen, however, this gas is consumed in larger quantities than would have taken place without the preliminary treatment with nitrogen. This process is spoken of as "going into oxygen debt."

The nerve can produce lactic acid from carbohydrate in the absence of oxygen. The lactic acid is oxidized very slowly. It is still uncertain as to whether the stimulation of a nerve involves a glycogen-lactic acid metabolism at all comparable to what takes place in muscle. One would expect that under the stimulation of oxygen, an increased amount of lactic acid would disappear, but the results do not support this view.

A somewhat mysterious effect is the production of ammonia when the nerve is stimulated. What the significance of this ammonia is, is not clear; nor has the origin of this compound been settled. There are two substances present in brain tissue which might give rise to ammonia: one is adenylic acid and the other is glutamine. Adenylic acid (p. 86) may lose ammonia, yielding inosinic acid. Here the change involved is a change of the adenine (in adenylic acid) to hypoxanthine (in inosinic acid).

Glutamine, also, may break down to glutamic acid and ammonia:

$$CONH_2.CH_2.CH_2.CHNH_2.COOH \rightarrow NH_3 + COOH.CH_2.CH_2.CHNH_2.COOH$$

There is reason to believe that glutamine itself is first formed by the reverse process: the combination of glutamic acid and ammonia. In any case, gray cortex can "bind" large quantities of ammonia provided glutamic acid is present; and, what is very significant, no other amino acid can take the place of glutamic acid.

As a result of stimulation in oxygen, this gas is consumed and carbon dioxide, ammonia and inorganic phosphate are liberated. The R. Q. (p. 316) is 0.9. Since it is unlikely that protein and fat are metabolized, the R. Q. should really be nearer 1, which is the R. Q. for carbohydrate in the living animal.

It is believed that potassium ions are involved in the electrical phenomena of nerve. Bathing a nerve in oxygenated sea water does not change the content of potassium in the nerve, despite the fact that the concentration of the element inside is ten times that of the element outside. Such a difference in concentration would give rise to a dif-

* Q = quantity of substance produced or consumed per milligram of dry weight of tissue per hour. A negative sign indicates absorption; a positive sign, evolution. The gas in question is expressed in cubic millimeters at N. T. P. For example, if the cortex of a rabbit's brain yields the figure $Q\frac{N_2}{CO_2} = +19$, it means that using the cortex (in nitrogen), 19 cu.μ or 19μl of carbon dioxide is produced per milligram of dry weight per hour. Where, as in the above case, $Q_{O_2} = -0.3$, it means that with the nerve under stimulation, 0.3 μl of oxygen, calculated at N. T. P., is consumed per milligram of dry weight per hour.

ference in electrical potential. But should the nerve be stimulated, or should it be deprived of oxygen, it begins to lose its potassium. It is of interest to find that so long as the potassium ions are retained by the tissue, so long can a potential difference be noted between the inside and the outside of the fiber. But the loss of potassium, resulting from stimulation, or oxygen deprivation, causes the potential difference to disappear.

The Metabolism of Brain.—Working with the intact brain is a very difficult problem. Despite difficulties, a number of facts have been gathered. The brain is apparently the only organ which gets its energy exclusively from the oxidation of carbohydrate. The reason for such belief is that the R. Q. of the brain *in situ* is 1 (Himwich and Nahum).

Human blood samples drawn from an artery and internal jugular vein simultaneously, permitting the estimation of the metabolic activity of the brain, showed the R. Q. of the brain to be 0.99. The data on the concentrations of sugar, lactic acid and oxygen in the blood entering and leaving the brain, add further to the belief that sugar is the principal source of energy in the brain. Even in diabetes, during which the R. Q. is low for the body, the R. Q. is not changed in so far as the brain is concerned. Its reserve of carbohydrate is almost negligible. It absorbs both glucose and lactic acid from cerebral blood.

Carbohydrate Metabolism.—Brain retains glycogen more tenaciously than liver. In this respect, brain resembles muscle. After pancreatectomy, the glycogen of the brain and the glycogen of the muscle remain high; this is not true of the glycogen of the liver.

On the other hand, the brain does not appear to store glycogen. The addition of glucose, with and without insulin, fails to raise the glycogen level.

Possibly both glycogen and the free fermentable sugar of brain are precursors of lactic acid.

Mammalian brain frozen *in situ* contains from 70 to 130 mg. of glycogen per 100 gm. The lactic acid content varies from 11.4 to 35.6 mg. per 100 gm.

Kerr has brought forward evidence for the presence of phosphocreatine in brain.

The difficulties of working with brain *in situ* have turned workers to the use of brain tissue slices—a method which, with all its shortcomings, is yielding results. For example, the lack of food reserves in the brain is made quickly apparent. This can be seen best by comparing different tissues. Slices of kidney, muscle or intact nerves can be suspended in Ringer's solution and the oxygen uptake measured. For a time this oxygen consumption continues at a fairly contant rate and then falls off. If slices of cerebral cortex are used, the rate of oxygen absorption diminishes almost from the start of the experiment. The reason for such failure is a lack of reserve food material (metabolite or suitable substrate). The addition of metabolite—glucose, mannose, fructose, lactate, glycerophosphate, pyruvate, etc.—will cause the oxygen consumption to continue at a steady rate for quite some time.

How important glucose and one or two closely related substances are to the normal function of the brain is seen in two instances: in the hepatectomized animal and in the animal suffering from "insulin shock." In both cases a hypoglycemia develops. In both cases, apparently as a result of the hypoglycemia, there develop weakness, sensations of hunger, mental confusion, delirium and, possibly, convulsions. In both cases the condition is relieved by an injection of glucose, or of mannose, or, more slowly and only with an intact liver, of fructose. Here the injection of lactate, pyruvate, succinate or glycophosphate is valueless.

The intact brain needs glucose and oxygen. It can, apparently, use mannose in the place of glucose; but fructose is useless in hepatectomy; suggesting that where fructose is of value—as in the normal animal—it is first changed to glucose in the liver. Both the glucose and the oxygen which are needed come from the blood.

It is also interesting to note that substances which are effective when dealing with brain tissue slices—lactate, pyruvate, etc.— are ineffective in hypoglycemia. "There is something special, from the point of view of the brain cells, about the metabolism of glucose," writes Holmes.

There is no strict parallelism between the oxidation of carbohydrates in brain and the oxidation in muscle; and yet there are similarities. Lactic acid, to be sure, is formed from glucose; and the intermediate compounds, temporarily attached to phosphate groups, are probably the same as in muscle metabolism (Chap. 16). Yet there remain grave doubts as to whether the glucose-lactic acid path is the only one used in connection with the metabolism of the brain.

With regard to possible similarities, it is quite true that minced brain or extracts of the brain reveal enzyme systems similar to those one finds in muscle extracts. Fructose diphosphate can be split into triosephosphate, glucose can be changed to phosphoglyceric acid and the latter to pyruvic acid: the coenzyme of brain is apparently the same as the coenzyme of muscle, etc.

On the other hand, whereas glucose is the substrate most readily acted upon by brain extract, glycogen is the one most favored by muscle extract. The conversion of glycogen to lactic acid is, according to our present knowledge, confined to one route only: via triosephosphate and catalyzed by adenosinetriphosphate. The conversion of glucose to lactic acid may take this route, or it may pass via the methylglyoxal, in the presence of reduced glutathione. In the brain—in contrast to muscle—the formation of lactic acid in the absence of phosphate is unaffected by the addition of adenosinetriphosphate, but the reaction is accelerated by the addition of reduced glutathione. Furthermore, it is claimed that brain forms lactic acid much more readily from methylglyoxal than from glucose, whereas muscle changes glycogen to lactic acid much more readily than it changes methylglyoxal to the acid.

Glutamic Acid.—We might dwell on glutamic acid for a little while because of its unique properties.

It is officially classified as a "non-essential" amino acid (p. 114), and yet it appears to be the only amino acid which is metabolized by brain tissue.

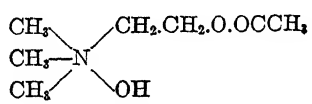
It is apparently unique in another unexpected way. The milder form of epilepsy, known as *petit mal*, characterized by brief lapse of consciousness, has now been successfully treated with glutamic acid, despite the fact—and this is another puzzling feature—that the amino acid is quite abundant in our food and can, for that matter, be easily synthesized by the body.

By the use of glutamic acid, the attacks in *petit mal*—sometimes reaching in number from 50 to 100 in a day—are reduced to 10 to 15 per cent of their initial frequency.

Correlated with this discovery of the relation of the amino acid to the mental behavior of the individual is the effect of glutamic acid—the *l*(+) form—in the maze-learning ability of rats, when measured by the number of trials and errors and the time required by the animal to run the maze. Rats receiving glutamic acid showed greater "learning ability." The results were "statistically significant."

Glutamic acid could not be replaced by glycine, so that whatever this property of glutamic acid is, it is not a property common to amino acids.

Acetylcholine and Sympathin.—In 1921 Loewi discovered that when the vagus nerve of a frog is stimulated, a choline-like substance is liberated from heart muscle Particularly through the work of Dale, the substance was later identified as acetylcholine, which, it was shown, was liberated at many junctions between conducting tissues. Stimula-



Acetylcholine.

tion of the nerve endings in voluntary muscle, inducing contraction, liberates acetylcholine. The parasympathetic nerves controlling involuntary muscle and secretory cells also liberate acetylcholine. On the other hand, the sympathetic fibers (also controlling involuntary muscles and secretory cells) liberate sympathin, according to Cannon—a substance which may, indeed, be identical with adrenaline itself. These two actions tend to balance one another: the one (parasympathetic) causes relaxation of muscle fibers, and the other (sympathetic) causes their contraction.

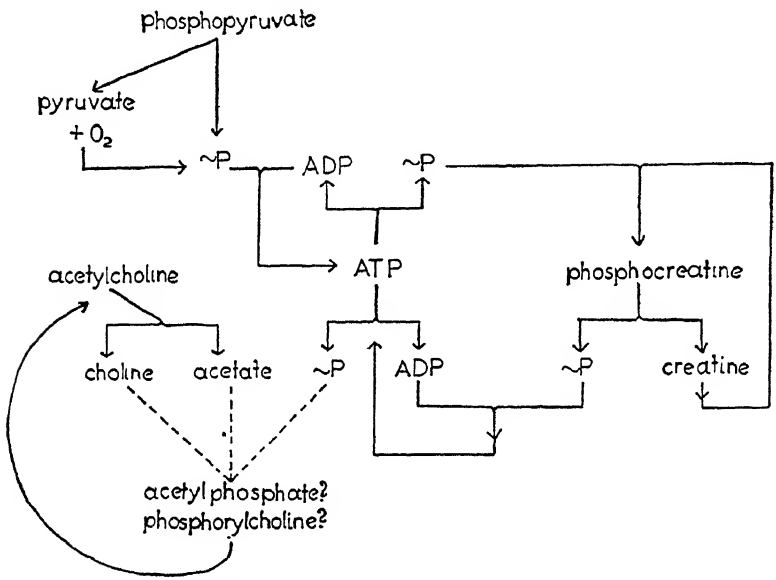
Through the work of Nachmansohn and others, much has been learned about the action of acetylcholine in its role in metabolic mechanisms. In the first place, an enzyme, *choline acetylase*, which helps in the synthesis of acetylcholine, can be extracted from rat brain and has also been obtained from the large electric organs of certain fish. This enzyme can function only in the presence of adenosine triphosphate (ATP) a supplier of "energy rich phosphate bonds." The loss of phosphate bonds in ATP is made up by the breakdown of

phosphocreatine, and the latter regains its phosphate group through the oxidation of dextrose (or pyruvic acid).

The cycle of operations to supply the energy needed for the synthesis of acetylcholine, then, is not unlike the reactions in muscle during the breakdown of carbohydrate.

Once acetylcholine is formed, its amount is controlled by the enzyme, *cholinesterase**—present in nervous tissue—which readily hydrolyzes acetylcholine.

The "acetylcholine" cycle is visualized in the following diagram (Fig. 142):



ATP = adenosinetriphosphate
ADP = adenosinediphosphate
~P = energy rich phosphate bond

Fig. 142.—The "acetylcholine cycle" (Nachmansohn, Cox, Coates and Machado, *J. Neurophysiology*, 6, 383.)

This cycle starts with the breakdown of acetylcholine. ATP yields one phosphate for the resynthesis of acetylcholine. The adenosine diphosphate (ADP) is immediately rebuilt to ATP by the breakdown of phosphorcreatine. The creatine is rephosphorylated by the breakdown of phosphopyruvic acid.

What is the origin of the acetyl group needed for the synthesis of acetylcholine? Its source may be some carbohydrate intermediate such as pyruvic acid or acetoacetic acid. In any case, the acetyl group is available in relatively large quantities in the body.

Significantly enough, Nachmansohn, using the electric organs of certain fishes, found that the energy needed for the resynthesis of

* There are said to be two esterases capable of hydrolyzing acetylcholine; a true choline esterase, acting exclusively on choline esters, and a more non-specific-enzyme, with under hydrolytic properties.

acetylcholine "was high enough to account for the electric energy released by the discharges, or impulses, in the electric organs."

There is reason to believe that glutamic acid (important in protein metabolism) and citric acid (equally important in carbohydrate metabolism), and possibly even other compounds, are coenzymes of chloine acetylase. The evidence is the following:

When active extracts of choline acetylase are dialyzed the activity rapidly decreases. In one test, whereas in the original extracts 40 to 80 gamma of acetylcholine are synthesized per gm. in 90 minutes, only 4 to 6 gamma are formed after a dialysis of two hours. If, now, *l*(+)glutamic acid, in concentration of 2×10^{-2} M, is added to the dialyzed extracts, the rate of formation of acetylcholine is increased 4 to 5 times. *d*(−)Glutamic acid has a small effect. Other amino acids tested have either no effect (*l*(+)aspartic, *dl*-serine) or less effect (*dl*-alanine, *dl*-methionine, glutamine). Among the dicarboxylic acids tried, *l*-malic, malonic and *α*-keto-glutaric show no effect, and succinic, a moderate effect. Only citric acid increases the activity of the enzyme comparable to that of glutamic acid.

Vitamins.—Aside from carbohydrate, such as glucose, a certain amount of material in brain was, for a time, classified as a reducing substance of noncarbohydrate origin. This now turns out to be none other than vitamin C (ascorbic acid) itself; this vitamin has been identified in various ways and has even been isolated. The cerebellum contains as much as 25 mg. per 100 gm. of tissue. It is generally believed that here, as elsewhere, the presence of vitamin C is not unrelated to the respiratory process.

As might be expected, the vitamin C content of the brain of a scorbutic guinea-pig is subnormal, the normal level being reached upon the addition of ascorbic acid to the diet.

On diets deficient in vitamin A there appear lesions in the brain and peripheral nerves. It is of interest, too, that vitamin A is a precursor of visual purple (Chap. 8).

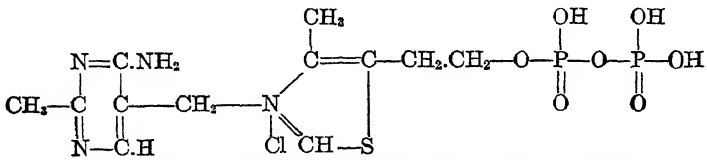
The metabolic defects on a vitamin B_1-deficient diet—which also appear elsewhere (heart and kidney) besides the nervous system—appear before there is any degeneration of tissue; which means that a fairly prompt supply of the missing factor restores the animal to normal health.

The development of beriberi is accompanied by an increase in the amount of pyruvate present in the brain and also by a decrease in the amount of oxygen consumed (Peters). It is assumed that the oxidation of glucose in the brain may follow the path: glucose → lactic acid → pyruvic acid → carbon dioxide, and it is believed that vitamin B_1 is concerned with the change of pyruvic acid, possibly a change to acetaldehyde and carbon dioxide. Yeast contains a carboxylase which converts α-keto acids to the corresponding aldehydes by loss of carbon dioxide:

$$CH_3.CO.COOH \rightarrow CH_3.CHO + CO_2$$

and it is believed that certain tissues, particularly the brain, also con-

tain such a carboxylase. It is further believed that a cocarboxylase is necessary for the proper functioning of the carboxylase itself, and that this coenzyme is none other than a pyrophosphoric ester of vitamin B_1 (Lohmann).



Cocarboxylase (vitamin B_1—or thiamin—pyrophosphate).

It is interesting to find in this connection that iodoacetate decreases the effectiveness of vitamin B_1 when added to an animal suffering from beriberi; however, if glutathione is added before the iodoacetate, the depressing effect of the latter is nullified.

From the work of Peters and his associates it seems clear that not only is carboxylase active in the oxidation of pyruvic acid in brain, but that the C_4 acids (succinic, fumaric, malic, oxaloacetic—see p. 327) play an essential rôle in this transformation. In other words, the C_4 acids are important whether we deal with oxidations in muscle or oxidations in brain.

Hormones.—We have already touched upon the subject of insulin in hypoglycemic shock, and we have also referred to the probability that nerves themselves liberate substances which may be regarded as hormones (p. 537). A study of one or two hormones, generated in other parts of the body, in their possible effect on the activity of the brain has been made. Thyroxine, for example, increases the respiration of both nerve and brain. This is presumably brought about by inducing an increased production of dehydrogenases, the enzymes involved in processes of oxidation.

Extracts from the adrenal cortex have a profound effect on the metabolism of sodium in the body (p. 427), and it is not surprising to find that they influence the potassium and sodium balance in the brain.

Effect of Drugs.—It has been emphasized that, from the point of view of metabolism, the brain is not a homogeneous tissue. Neither is it homogeneous in its anatomical or chemical structure. The multiplicity of results obtained by the use of different chemical is not surprising.

To cite a few examples: cocaine paralyzes sensory nerve endings; atropine paralyzes the nerve endings of the parasympathetic system only; morphine depresses the centers dealing with pain perception; analgesics and antipyretics (salicylates, aspirin, etc.) depress the pain-perceiving and temperature-regulating mechanisms; alcohol depresses the power of judgment and releases inhibitions; etc.

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This chapter is based on Chap. 10 of Holmes' *The Metabolism of Living Tissue* (1937)—a most suggestive volume. Other rich sources of information are the articles by Gerard, *Ann. Rev. Biochem.*, **6**, 419 (1937); and by Quastel, *Ann. Rev. Biochem*, **8**, 435 (1939), *Physiol Rev.*, **19**, 135 (1939).

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See, also, the following: Needham and Green, *Perspectives in Biochemistry* (1937), pages 296–307 (biochemistry and mental disorders, by Quastel), and pages 308–317 (biochemistry and the function of the nervous system, by Holmes); Brown, *Physiol Rev*, **17,** 485 (1937) (transmission at nerve endings by acetylcholine), Rosenbluth, *Physiol, Rev.*, **17,** 514 (1937) (transmission of sympathetic nerve impulses).

What appear as two classical addresses we owe to Loewi, *Harvey Lectures* (1932–1933), p. 218 (humoral transmission of nervous impulse); and Dale, *Harvey Lectures* (1936–1937), p 229 (transmission of nervous effects by acetylcholine).

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Papers dealing with lipid metabolism in brain are Sperry, Waelsch and Stoyanoff, *J. Biol. Chem.*, **135,** 281 (1940); Waelsch, Sperryand Stoyanoff, *Ibid.*, **135,** 291 (1940); Fries and Shaikoff, *Ibid.*, **141,** 479 (1941).

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Experiments dealing with the phospholipid activity of the brain are recorded by Fries, Schachner and Chaikoff, *J. Biol. Chem.*, **144,** 59 (1942).

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For the extraordinary effects of glutamic acid on brain metabolism, see Price, Waelsch and Putnam, *J. Am. Med. Assoc.*, **122,** 1153 (1943); Waelsch and Price, *Archives of Neurology and Psychiatry,* **51,** 393 (1944); *Nutr. Rev.*, **3,** 135 (1945).

APPENDIX

THE NUTRITIVE VALUE OF FOODS. (Newburgh and Mackinnon, *The Practice of Dietetics*. By permission of The Macmillan Company, Publishers.)

Food, 100 gm.	Protein N × 6.25. gm.	Lipids. gm.	Carbohydrates. Available. gm.	Carbohydrates. Indigestible. gm.	Calories.	Ca. gm.	P. gm.	Fe. mg.
Almonds	21.0	54.9	6.5	7 3	604	0.239	0.465	4.07
Apples	0 3	0.4	7.7	2.2	36	0.007	0.012	0.43
Apricots	1 0	0 1	4.6	1.6	21	0.014	0.025	1.45
Artichokes, Jerusalem	2 2	0.1	15.8	1.0	73	?	?	1.9
Asparagus	2.2	0.2	2.4	1 5	20	0.025	0.039	0.79
Avocado	2.0	23.2	6.7	2.5+	243	?	?	0.28
Bacon	10.5	64.8	..	..	625	0.006	0.108	?
Banana	1.2	0.2	18.7	1.5	82	0.009	0.031	1.76
Beans, lima, dry	18.5	1.5	65.9	?	349	0.071	0.338	11.66
Beans, navy, dry	22.5	1 8	55.2	4.4+	327	0.160	0.471	9.52
Beans, soy, dry	36.7	18.2	26.6	5.1+	417	0.206	0.580	0.78
Beef, round, lean	19.7	8.0	..	..	151	0 011	0.212	4 1
Beef, roast, fat	22.3	28.6	.	..	347	0 013	0.240	4.9
Beet greens	2.0	0.3	4.2	1.4+	27	?	?	3.13
Blackberries	1.2	1.1	4.9	5.9	34	0.017	0.034	0.91
Blueberries	0.6	0.6	13.9	1.2+	63	0.020	0.008	0.80
Bran	16.4	6.1	12.0	5.3	169	0.120	1 215	8.52
Brazil nuts	16.8	69.4	2.5	4.6	705	?	?	3.93
Bread, graham	8.9	1.8	51.0	1.1+	256	0.050	0.218	1.0?
Bread, rye	9.0	0.6	52.7	0.5+	252	0 024	0.148	0.5
Bread, white	9.3	1.2	52.2	0.5+	257	0.027	0.093	0.22
Bread, whole wheat	9.7	0.9	48.5	1.2+	242	0.05	0.175	1.6?

THE NUTRITIVE VALUE OF FOODS—*Continued*

Food, 100 gm.	Protein N × 6.25. gm.	Lipids. gm.	Carbohydrates. Available. gm.	Carbohydrates. Indigestible. gm.	Calories.	Ca. gm.	P. gm.	Fe. mg.
Broccoli	3 3	0 2	4 2	1 3+	32	?	?	1.4
Brussels sprouts	4 4	0.5	6 3	2.6	47	0.027	0 120	2.23
Butter	1.0	85.0	..	..	769	0.015	0.017	?
Buttermilk	3 0	0.5	4.8	0.0	38	0.105	0 097	?
Cabbage, white	1.4	0.2	3.0	2.3	20	0.045	0 029	0.34
Cantaloupe	0.6	0.2	5.1	0.7+	25	0.017	0 015	0 51
Carrots	1.2	0.3	7 0	2.3	35	0.056	0.046	1.07
Cauliflower	2.4	0.2	2 8	2 2	22	0.123	0.061	1.43
Celery	1.3	0.2	1 0	1.5	11	0.078	0.037	0 77
Cheese, cheddar	27.7	36.8	4 1	0.0	458	0.930	0.683	1.38
Cheese, cottage	20.9	1.0	4.3	0.0	110	0.077	?	?
Cherries, sweet	1.1	0.5	16.4	1.4	74	0.019	0.31	0.51
Chestnuts	6.2	5 4	29.2	3.8	190	0.034	0.093	4.10
Chicken, broiler	21.5	2.5	..	..	108	0.012	0.232	0.70
Chicken, fowl	19.3	16.3	..	..	234	0.011	0.208	0.70
Chocolate, milk	8.0	35.0	51 0	?	551	?	?	?
Cocoa	21.6	28.9	37.7	0.0	497	0.112	0 709	3.13
Cocoanut, shredded	3.7	34.0	4.8	13.2	340	0.035	0 09	2.67
Cod, salt	25.4	0.3	..	..	104	0.028	0.292	?
Cod liver oil	..	100.0	..	..	900	?	?	?
Corn, sweet	3.7	1.1	21.9	0.9+	102	0.006	0.103	0.47
Cornstarch	..	..	90 0	..	360			
Corn syrup	..	..	75.0	..	300			
Crabmeat	15.8	1.5	0.7	..	79	0.17	0.181	?
Crackers, graham	10 0	9.4	72.3	1.5+	414	0.024	0.203	1.88
Crackers, saltines	10.6	12.7	68.0	0.5	429	0.022	0.102	?

THE NUTRITIVE VALUE OF FOODS—*Continued*

Food, 100 gm.	Protein N × 6.25. gm.	Lipids. gm.	Carbohydrates. Available. gm.	Carbohydrates. Indigestible. gm.	Calories.	Ca. gm.	P. gm.	Fe. mg.
Cranberries	0.4	0.7	9.0	2.3	43	0.018	0.013	0.44
Cream, 20 per cent	2.9	20.0	4.0	..	208	0.097	0.086	0.13
Cream, 40 per cent.	2.2	40.0	3.0	..	381	0.086	0.067	0.10
Cucumber	0.7	0.1	1.75	0.8	11	0.016	0.033	0 33
Currants, red	2 4	0.4	3.7	4.0	28	0 026	0 038	0.63
Dates, dry	2 1	2.8	78.4	?	347	0.065	0 056	9.11
Duck	22 3	3.3	..	..	119	0.013	0.240	1.71
Eggs, hen's	13.4	10.5	..	..	148	0.067	0.180	2.52
Egg white	12 3	0 2	..	..	51	0.015	0.014	?
Egg yolk	15 7	33 3	..	..	362	0 137	0.524	7.60
Eggplant	1.6	0.2	2 4	1 9	18	0.011	0.34	0.60
Figs, fresh	1 4	0.4	6.9	4 0	37	0.053	0.036	0.79
Figs, dried	4 3	0.3	23.3	16.4	113	0.162	0 116	3.96
Flour, buckwheat	6.4	1.2	77.5	0.4+	346	0.010	0 176	3.20
Flour, rye	6.8	0.9	78.3	0.4+	349	0 18	.289	2 83
Flour, white	11.2	1 0	74.7	0.2+	353	0 020	0.092	0.91
Flour, whole wheat	13.8	1.9	71.0	0.9+	356	0.031	0.238	?
Gelatin	91.4	0.1	..	..	366	?	?	?
Goose, young	16.3	36.2	..	..	391	0.009	0.176	2.02
Gooseberries	0.8	0.4	5.3	3.3	28	0.035	0.031	0.47
Grapes	1.4	1.4	10.0	1.1	58	0 019	0.031	0.82
Grape juice, concord.	0.3	..	17.3	..	70	0.011	0.011	?
Grapefruit	0.5	0.2	5.8	1.4	27	0.021	0.020	0.27
Halibut	18.6	5.2	..	..	121	0.020	0.214	0.93
Ham, fresh, lean	25.0	14.4	..	..	230	0.014	0.269	?
Ham, smoked, med. fat	16.3	38.8	..	..	414	0.009	0.176	?

THE NUTRITIVE VALUE OF FOODS.—*Continued*

Food, 100 gm.	Protein N × 6.25. gm.	Lipids. gm.	Carbohydrates. Available. gm	Carbohydrates. Indigestible. gm.	Calories.	Ca. gm.	P. gm.	Fe. mg.
Hazelnuts	15.6	65 3	7.3	2 1+	611	0 287	0 354	4.50
Hickory nuts	15.4	67.4	11.4	?	714	?	?	2 38
Honey	0.4	.	81.2	..	326	0 004	0 019	1 15
Kidney, veal	16 9	6.4	..	..	125	0 010	0.182	?
Lamb chops	18.7	28.3	..	..	329	0 011	0 202	?
Lamb roast	19.7	12 7	..	.	193	0 011	0 212	?
Lemon juice		..	2.0	?	8	0 024	0 010	0 15
Lentils, dry	25 7	1.0	59.2	?	349	0 107	0 438	?
Lime juice	0 5	..	7.8	?	33	?	?	?
Liver, beef	20 4	4 5	..	.	126	0 012	0 220	8.3
Liver, calf	19 0	5 3	..	..	124	0 011	0 205	5.4
Lobster	18 1	1 1	0.5	.	84	0 020	0.208	0 44
Mackerel	18 7	7 1	..	.	139	0 020	0.215	0 75
Milk, whole	3.3	4.0	5.0	..	69	0 120	0 093	0 15
Milk, skim	3 4	0.3	5 1	..	37	0 122	0 096	0 15
Milk, condensed, sweetened	8 8	8.3	54.1	..	326	0 300	0 235	?
Milk, evaporated	9 6	9 3	11.2	..	167	?	?	0 44
Milk, human	0 98	4 4	6.9	..	71	0.028	0 014	?
Molasses	2.4	.	69.3	.	287	0 211	0 44	7.97
Oats, rolled	16.1	7.2	66.6	0 9	396	0 069	0 392	3.80
Olives, green	0 8	20.2	0.0	1 3	185	0.122	0 140	2.11
Onions	1.4	0 2	9.0	2 0	43	0.034	0 045	0.30
Oranges	0 9	0 2	6.2	1 8	30	0.045	0.021	0.60
Orange juice	0.6	..	6.1	0 0	27	0.029	0.016	0.42
Peaches	0.5	0.1	6.7	1.6	29	0.16	0.024	0.36

THE NUTRITIVE VALUE OF FOODS—*Continued*

Food, 100 gm.	Protein N × 6.25. gm.	Lipids. gm.	Carbohydrates		Calories.	Ca. gm.	P. gm.	Fe. mg.
			Available. gm.	Indigestible. gm.				
Peanuts	25 8	38.6	11.4	6.3	496	0 071	0 399	2 31
Peas, green	6 7	0.4	12 7	5 0	81	0 028	0 127	1.77
Pecans	11.0	71 2	13.3	?	738	0 089	0 335	2.58
Pineapple	0 4	0.2	9 9	1.7	43	0 018	0 028	0.32
Plums	0 7	0 2	5.4	1.5	25	0.020	0.032	0.77
Pork chops, lean	20 3	19 0	..	..	252	0.012	0 219	?
Pork chops, med. fat	16.6	30.1	..	..	337	0.010	0.179	?
Potato, white	2 0	0 1	18.7	0 4	84	0 140	0 058	0.85
Potato, sweet	1 8	0 7	26.9	1.0	121	0.019	0.045	0.92
Prunes, dried	2 1	..	71.2	4 5	293	0.054	0.105	5.17
Radishes	1 2	0.1	1.6	1 1	12	0.021	0 029	1.36
Raisins (seedless)	2 6	3.3	76 1	1 0	344	0 064	0 132	4.13
Raspberries, red	1 1	0 6	2.7	4.2	21	0.049	0 052	0.99
Rhubarb	0 5	0 1	2 6	1 2	13	0.044	0.031	0.86
Rice, polished	8.0	0 3	78.8	0 2	350	0.009	0.096	1 05
Salmon	22 0	12.8	..	..	203	0.024	0.253	0.83
Spinach	2.3	0.3	1.6	1.6	18	0.067	0.068	6.60
Squash, summer	0 6	0 1	3.4	0 5	17	0 018	?	0.89
Squash, winter	1 5	0 3	7.4	1 4	38	0.018	?	0.55
Strawberries	0 8	0 6	4 4	1 2	26	0.041	0.028	0 66
Tangerines	0 8	0.3	9.9	1 0	45	?	?	0.60
Tomatoes	1 0	0 3	2.4	1.1	17	0.011	0.026	0.60
Trout	17 8	10.3	..	..	164	0.019	0 204	0 78
Turkey, dark meat	21 4	20 6	..	..	271	0.012	0 231	2 04
Turkey, light meat	25.7	9.4	..	..	187	0.015	0.277	1 03
Turnips	1.1	0.2	3.5	1.6	20	0.064	0.046	0.70

THE NUTRITIVE VALUE OF FOODS—*Continued*

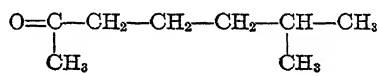
Food, 100 gm.	Protein N × 6.25. gm	Lipids. gm.	Carbohydrates. Available. gm.	Carbohydrates. Indigestible. gm.	Calories.	Ca. gm.	P. gm.	Fe. mg.
Veal chops, med. fat	19.9	10.8	..	..	177	0.012	0.215	?
Walnuts, black . ..	27 6	56 3	6.0	5.7	641	?	?	5.98
Walnuts, English ..	18 4	64.4	5.0	5.4	673	0 089	0 358	2.14
Water cress	1 7	0.3	0.7	1.0	8	0.187	0.005	7.21
Watermelon.	0 5	0 2	2.5	0.9	14	0.011	0.003	0.23
Whitefish.....	22 9	6.5	..	..	150	0.150	0.263	0.42

EVIDENCE FOR THE CHOLESTEROL STRUCTURE (refers to p. 39)

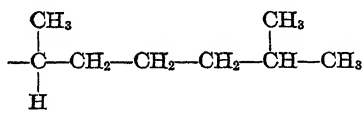
The complete evidence for the cholesterol structure involves complicated chemical reactions However, an approach to the solution of the problem may be given at this point. The hydroxyl group in cholesterol can be converted to a keto group by gentle oxidation, suggesting the presence of a secondary alcohol Various esters of cholesterol, involving this hydroxyl group, have been prepared. That a double bond is present may be deduced by the behavior of the compound towards bromine, hydrogen, and the halogen acids Saturated compounds are formed in each case. The double bond can be saturated (with hydrogen, in this case), and by suitable means the hydroxyl group can be removed, leaving a compound with the formula $C_{27}H_{48}$, known as *cholestane.*

For this cholestane to be a *paraffin* hydrocarbon, eight more hydrogen atoms would be needed. This suggested a possibility that the substance is made up of four saturated rings

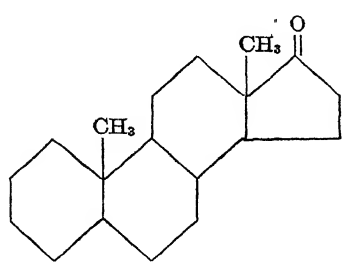
What these rings are can be deduced in the following way: when cholestane is treated with chromic acid a ketone, which can be steam-distilled, is isolated, with the formula



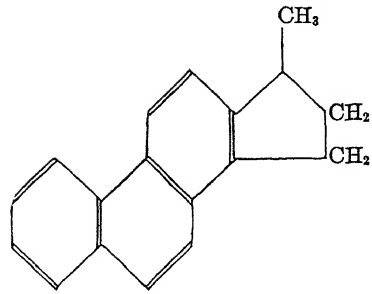
which is methylisohexylketone. From this reaction it is inferred that cholesterol contains a side chain with the following formula:



Another product isolated from the reaction with chromic acid is a ketone, $C_{19}H_{30}O$ which would conform to the formula,

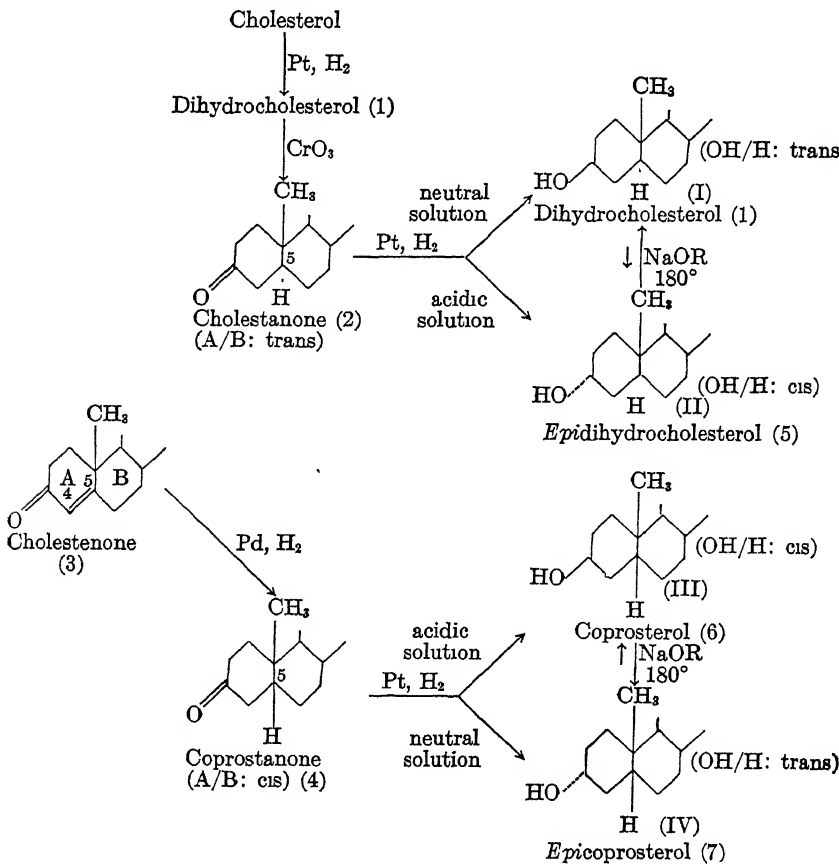


The nature of the four rings in cholesterol may be obtained by treatment with selenium at 320° C.—a dehydrogenation process which converts the saturated rings into unsaturated (aromatic) rings. The formula for the product obtained turns out to be $C_{18}H_{16}$. Its constitution was established by synthesis. It is methylcyclopentanophenanthrene,

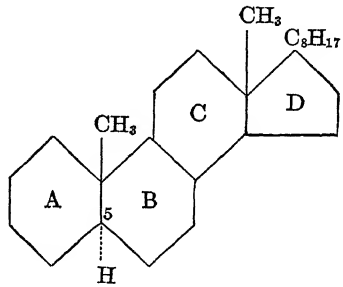


which means that cholesterol has three six-membered rings, connected as in phenanthrene, and one five-membered ring.

A few notes with regard to the stereochemistry of the cholane series are appended. When cholesterol is hydrogenated, dihydrocholesterol or β-cholesterol (1) is obtained This substance on oxidation gives the ketone cholestanone (2). If, on the other hand, cholesterol is first oxidized to cholestenone (3) and this ketone reduced, an isomer of cholestanone results to which the name coprostanone (4) is given Coprostanone (4) and cholestanone (2) differ only in the orientation of the groups attached to carbon atom 5.

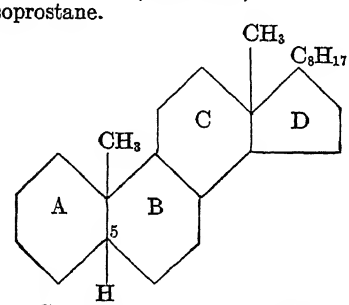


The stereochemical configurations of the four dihydrocholesterols are shown in (1), (5), (6) and (7). (1) and (5) are derived from cholestane,



Cholestane.
(A/B. trans type)
(Allo series)

and (6) and (7) from coprostane.



Coprostane (Pseudocholestane).
(A/B: cis type)
(Normal series)

SOME PROPERTIES OF SOLUTIONS*

Cells contain much water; more than two-thirds the weight of the body is water. In this medium there are inorganic and organic substances, such as salt and sugar (which form solutions), and more complex organic substances, such as proteins, which form colloidal particles. To some extent, such gases as oxygen and carbon dioxide are also dissolved in water.

Body fluids like blood, lymph, digestive juices, urine, milk, represent varying types of solutions.

An understanding of some fundamental facts concerning solutions is essential in biochemistry. Such facts, conveniently classified under physical chemistry, may be presented as a prelude to the chapters on proteins and enzymes, which have been studied both from the angle of the physical chemist and from that of the organic chemist.

While such facts as are brought out in this chapter find their application in every field of biochemistry, their usefulness is strikingly illustrated in studies dealing with the chemistry of respiration (Chap. 15).

The theory of electrolytic dissociation, from which so much of value follows, is based upon several properties of solutions which will now be discussed.

Vapor Pressure.—When a liquid evaporates the escaping molecules exert a pressure known as the vapor pressure. In a mixture, each component exerts its independent pressure. When one mole of methyl alcohol is added to 4 moles of ethyl alcohol, the partial pressure of methyl alcohol is $\frac{1}{5}$ of the vapor pressure of pure methyl alcohol (at that temperature), and the partial pressure of ethyl alcohol is $\frac{4}{5}$ of the vapor pressure of pure ethyl alcohol. The total pressure of the solution is the sum of these two partial pressures.

The vapor pressure of either component is lowered on the addition of the other. This is particularly important when we have a solution of a non-volatile solute, such as sugar, in a liquid such as water. The sugar is non-volatile and, for practical purposes, has zero vapor pressure. Therefore the partial pressure of the water in the solution is the total vapor pressure of the solution. The vapor pressure of the solution is therefore less than the vapor pressure of the pure solvent.

For example, if one mole of sugar is mixed with 99 moles of water, the vapor pressure of the solution is $\frac{99}{100}$ of the vapor pressure of pure water (at that temperature). This is an example of a law discovered by Raoult.

Boiling Point.—A liquid boils when its vapor pressure equals that of the atmosphere. Since the atmospheric pressure is normally 760 mm., the boiling point of water is 100° C., because at that temperature the vapor pressure is 760 mm. When the external pressure falls to 733 mm., water boils at 99° C.

*Refer to Chap. 4.

A non-volatile solute lowers the vapor pressure of a solvent. Now the boiling point is that temperature at which the vapor pressure of the liquid reaches the external pressure; therefore solutions will not boil at the same temperatures as the pure liquids, but will require a higher temperature.

Freezing Point.—The freezing point is defined as that temperature at which the liquid is in equilibrium with the solid. A solution, with a lower vapor pressure than the pure solvent, will not be in equilibrium with the solid solvent at the *normal* freezing point. The system must be cooled to that temperature at which the solution and the solid solvent have the same vapor pressure. The freezing point of a solution is therefore less than that of the pure solvent.

Osmosis.—If two open vessels, one containing pure water and the other an aqueous solution, are covered with a bell jar, the pure water gradually passes through the vapor state into the solution until it is all gone. The reason for this is that the solution has a smaller vapor pressure than the pure solvent.

If now the two vessels are so arranged that the liquids are in contact through a membrane permeable to the solvent but not to the solute, we find that the solvent passes through this semi-permeable membrane into the solution. The number of impacts of water molecules per sq. cm. is greater on the pure solvent side than on the solution side, and hence one would anticipate a flow of solvent into the solution. This flow is known as *osmosis.*

Osmosis takes place whenever a solution is separated by a semi-permeable membrane from a solution of different concentration. In osmosis the solvent flows from the less concentrated solution into the more concentrated solution.

Osmotic Pressure.—If the solvent is to be prevented from penetrating the semi-permeable membrane (see preceding paragraph) pressure has to be applied to the solution. This pressure is equivalent to the osmotic pressure. The osmotic pressure of a dilute solution is proportional to the temperature and to the fraction of solute molecules in solution.

The Theory of Electrolytic Dissociation.—Aqueous solutions of acids bases and salts are excellent conductors of electricity. These substances, "electrolytes," constitute a large group of compounds. Sugar, alcohol, glycerol, and, in general, compounds of carbon ("organic" compounds) are poor conductors of electricity; they are the "non-electrolytes."

Of course, as might be anticipated, in between the excellent conductors and the poor conductors there are many gradations.

Good conductors of electricity give rise to abnormal boiling point elevations, abnormal freezing point depressions and abnormal osmotic pressures. The poor conductors give normal values.

To explain these phenomena, Arrhenius proposed his now celebrated theory of electrolytic dissociation. He suggested that substances which when dissolved in water are good conductors, are *dissociated.* Sodium chloride, for example, becomes

$$NaCl \rightleftarrows Na^+ + Cl^-$$

sodium, electrically charged (a sodium *ion*) and chlorine, electrically charged (a chloride *ion*). In terms of the effects on boiling point, freezing point and osmotic pressure, each ion behaves as if it were a molecular unit; so that when sodium chloride is dissolved, it behaves as if it were two ions ($Na^+ + Cl^-$) rather than one molecule. Hence the abnormal results.

On the other hand, non-electrolytes dissociate very little. For example, cane sugar when dissolved in water would still remain as cane sugar molecules. Cane sugar solutions would therefore show no abnormal changes in boiling point, freezing point and osmotic pressure.

From our knowledge of the structures of the atom and the molecule, we know sodium chloride to be completely ionized not only in dilute solutions but also at higher concentrations. The salt is in the completely ionized state.

We can therefore rewrite Arrhenius' equation thus:

$$Na^+Cl^- \rightleftarrows Na^+ + Cl^-$$

which means that when salt is dissolved in water, or when a concentrated solution is diluted, the principal effect is an electrolytic dissociation of *pre-existing ions* and *not* an ionization of pre-existing molecules.

Acids and Bases.—The classical view—the view based on the work of Arrhenius—is that an acid yields hydrogen ions in solution and a base yields hydroxyl ions in solution. In dealing with aqueous systems, this view is still a convenient one for many purposes and will be freely used throughout the book. However, the more modern theory, due to Bronsted (and others), has wider implications. According to Bronsted, an acid is a substance which gives off protons (hydrogen ions), and a base is one which unites with protons. In general,

$$A \rightleftarrows H^+ + B$$

A representing the acid and B the base.

Here the definition of an acid remains essentially the same as before, but the definition of a base is less restricted. Formerly the definition of a base was formulated with the basis that water is the solvent, but of course there can be many solvents other than water. For instance, in the equation

$$HCl \rightleftarrows H^+ + Cl^-$$

HCl was considered an acid because in water solution it yielded H^+. The newer concept may be written

$$\underset{\text{(acid)}}{HCl} \rightleftarrows H^+ + \underset{\text{(base)}}{Cl^-}$$

And again,

$$\underset{\text{(acid)}}{CH_3COOH} \rightleftarrows H^+ + \underset{\text{(base)}}{CH_3COO^-}$$

$$\underset{\text{(acid)}}{H_2CO_3} \rightleftarrows H^+ + \underset{\text{(base)}}{HCO_3^-}$$

$$\underset{\text{(acid)}}{H_3O^+} \rightleftarrows H^+ + \underset{\text{(base)}}{H_2O}^*$$

* The H^+ exists in aqueous solution in the hydrated form, H_3O^+, the "hydronium" or "oxonium" ion.

These acids yield protons (H^+) and particles which combine with protons—particles which, by definition, are bases.

An acid, then, is a substance which yields a proton and a base; and a base is a substance which combines with a proton to form an acid.

Amphoteric Substances.—When a substance acts as an acid and may also act as a base, it is known as an "amphoteric substance." Water, for example, may act as an acid because it yields protons:

$$HOH \rightleftarrows H^+ + OH^-$$

and it may act as a base because it combines with protons:

$$HOH + H^+ \rightleftarrows H_3O^+$$

Liquid ammonia may act as an acid:

$$NH_3 \rightleftarrows H^+ + NH_2^-$$

and also as a base:

$$NH_3 + H^+ \rightleftarrows NH_4^+$$

Dissociation of Water.—While in general chemistry, a discussion of acids and bases must include solvents other than water, in biochemistry, dealing with body tissues, water is the fluid of the utmost importance.

Returning to the classical, and from a practical point of view, the more convenient form for the student, water dissociates thus:

$$HOH \rightleftarrows H^+ + OH^{-*}$$

Applying the law of mass action,

$$K = \frac{[H^+][OH^-]\dagger}{[HOH]}$$

or

$$K[HOH] = [H^+][OH^-].$$

Water dissociates but slightly (one molecule in ten million), which means that the concentration of undissociated water remains practically constant.

Therefore, for K[HOH] we can substitute a new constant, K_W, and write

$$K_W = [H^+][OH^-]$$

where K_W stands for the "ion product" or "dissociation constant" of water.

We further learn from this equation that the product of $[H^+]$ and $[OH^-]$ is a constant. K_W at 25° C. is 1×10^{-14}; so that, since

$$K_W = [H^+][OH^-],$$

and $[H^+]$ is equal to $[OH^-]$

$$K_W = [H^+]^2 \text{ or } [OH^-]^2$$

or $[H^+]$ or $[OH^-] = \sqrt{K_W}$.

So that

$$[H^+] = \sqrt{K} = \sqrt{1 \times 10^{-14}} = 10^{-7} \text{ (moles per liter)}$$

* Or, more correctly, $2H_2O \rightarrow H_3O^+ + OH^-$.

† [ ] = moles per liter.

The Meaning of *p*H.—*p*H is the negative logarithm of $[H^+]$, or the logarithm to the base 10 of the reciprocal of $[H^+]$.*

$$p\text{H} = -\log [H^+] = \log \frac{1}{[H^+]}$$

and

$$[H^+] = 10^{-p\text{H}}$$

So that if $[H^+] = 10^{-7}$, then $p\text{H} = 7.0$

if $[H^+] = 10^{-1}$, then $p\text{H} = 1.0$

In other words, the greater the hydrogen ion concentration, the lower the *p*H; so that a solution with a *p*H of 2 is more acid than one with a *p*H of 6.

As an example of an interconversion, if $[H^+] = 2.73 \times 10^{-4}$, what is the *p*H?

Log 2.73 = 0.43 and log $10^{-4} = -4$

$$p\text{H} = \log \frac{1}{2.73 \times 10^{-4}} = \log 1 - \log (2.73 \times 10^{-4})$$

Log 1 = 0, then

$$p\text{H} = -\log (2\ 73 \times 10^{-4})$$
$$= -\log 2.73 - \log 10^{-4}$$
$$= -0\ 43 + 4$$
$$p\text{H} = 3.57$$

If the *p*H is 5.41, what is $[H^+]$?

$$[H^+] = 10^{-p\text{H}}$$
$$= 10^{-5\ 41}$$
$$= 10^{-6+0\ 59}$$
$$= 10^{0.59} \times 10^{-6}$$

Antilog of 0.59 = 3.89

Therefore $[H^+] = 3.89 \times 10^{-6}$

Some examples of *p*H and $[H^+]$ are given in approximate figures:

Substance	*p*H	$[H^+]$
N HCl	0 0	1 0
0.1 *N* HCl	1 0	10^{-1}
0.01 *N* HCl	2 0	10^{-2}
N CH_3COOH	2.3	$4\ 3 \times 10^{-3}$
0.1 *N* CH_3COOH	2 8	$1\ 3 \times 10^{-3}$
N NaOH	14 0	10^{-14}
0.1 *N* NaOH	13 0	10^{-13}
N NH_4OH	11 7	1.7×10^{-12}
0.1 *N* NH_4OH	11 2	$5\ 4 \times 10^{-12}$

Dissociation of a Weak Acid in the Presence of Its Salt.—In the body we find carbonic acid in the presence of its salt, sodium bicarbonate, and, in general, weak acids in the presence of their salts.

In the dissociation of a weak acid, $HA = H^+ + A^-$, by applying the mass action law we get the expression

$$\frac{[H^+] \times [A^-]}{[HA]} = K_a \quad (1)$$

* For the sake of simplicity, we adhere here to the "hydrogen ion," $[H^+]$ rather than to the more modern "hydronium ion," $[H_3O^+]$.

where K_a = the dissociation constant of the acid. Water, a weak electrolyte, gives us the expression

$$\frac{[H^+] \times [OH^-]}{[H_2O]} = K$$

But $[H_2O]$ (undissociated water) is very large and practically constant; so that

$$[H^+] \times [OH^-] = K_W$$

K_W being the dissociation constant of water. K_W at 22° C. is $\frac{1}{10^{14}}$.

$$[H^+] = [OH^-] = 10^{-7}. \quad \text{Or } p\text{H} = 7.$$

We can titrate an acid with alkali, determining at intervals the *p*H of the solution (by methods to be outlined presently), and we get a titration curve of the type shown (Fig. 143) which represents the reaction of acetic acid with sodium hydroxide. The curve is S-shaped (sigmoid). The curve changes least when the acid is half neutralized. At this point the solution shows its maximum buffering effect; a **buffer** being a solution which resists change of *p*H when acid or alkali is added.

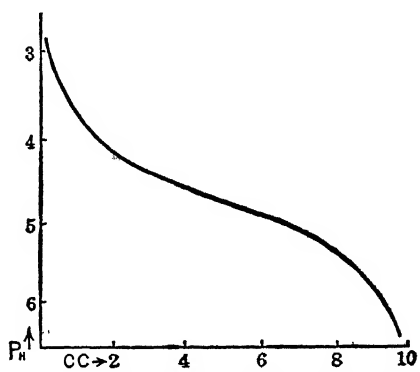


Fig 143.—Titration curve of acetic acid and sodium hydroxide (Reprinted by permission from Scarth and Lloyd, *Elementary Course in General Physiology*, John Wiley and Sons, Inc., Publishers)

From (1), we get

$$[H^+] = K_a \frac{[HA]}{[A^-]}$$

In the presence of a weak acid (acetic) and its salt, the acid is very slightly dissociated, and the anions are approximately all due to the salt. The [HA], then, represents (approximately) total acid, undissociated and dissociated; and the $[A^-]$ represents (approximately) total salt, undissociated and dissociated. So that we can write

$$[H^+] = K_a \frac{[\text{Acid}]}{[\text{Salt}]}$$

Transforming this into pH value (the usual form of the Hasselbach-Henderson equation), we get

$$p\text{H} = p\text{K}_\text{a} + \log \frac{[\text{Salt}]}{[\text{Acid}]}$$

$$\text{where } pk_\text{a} = -\log k_\text{a}$$

Where we have maximum buffering action (when [salt] = [acid]), then

$$pH = pk_\text{a}$$

Methods of Determining pH.—There are two common methods available: the indicator method and the method involving the use of the potentiometer. The details involved in such determinations must be left to texts devoted to laboratory methods; but a few general remarks will be made.

Indicators, with which pH is often measured, change colors with change of pH. These substances are usually weak acids or bases wherein the dissociated ion and the undissociated molecule have different colors; or the one has color and the other has none. The color changes take place within certain pH ranges, different indicators having different pH ranges.

The dissociation curves of several indicators are shown in Fig. 144; and it will be noticed that these curves resemble the curve shown in Fig. 143.

A list of several indicators, giving changes in acid and alkaline solutions, is given in the accompanying table.

A Few Common Indicators. (Bertho and Grassmann, *Laboratory Methods of Biochemistry*)

Common name.	Zone of color change.	Color in	
		Acid solution	Alkaline solution
Thymol blue*.	1 2– 2 8	red	yellow
Bromophenol blue	3 0– 4 6	yellow	blue
Methyl orange	3 1– 4 4	red	yellow
Methyl red	4 0– 6 0	red	yellow
Bromocresol purple	5 2– 6 8	yellow	violet
Bromothymol blue	6 0– 7 6	yellow	blue
Phenol red	6 8– 8 4	yellow	red
Naphtholphthalein	7 3– 8 7	faint pink	blue
Cresol red	7 2– 8 8	yellow	red
Cresolphthalein	8 2– 9 8	colorless	red
Phenolphthalein	8 3–10 0	colorless	red
Alizarin yellow R	10 3–11 7	yellow	red

* Thymol blue exhibits, in addition, a second zone of color change between 8.0 and 9 6 (yellow—blue) Cresol red and phenol red become pink at pH less than 2 besides changing in the zone shown in the table.

Aside from the indicator method of determining pH, there is the method which involves **electrometric measurement,** which, though not so simple to carry out, is more accurate.

A metal immersed in the solution of one of its salts gives rise to

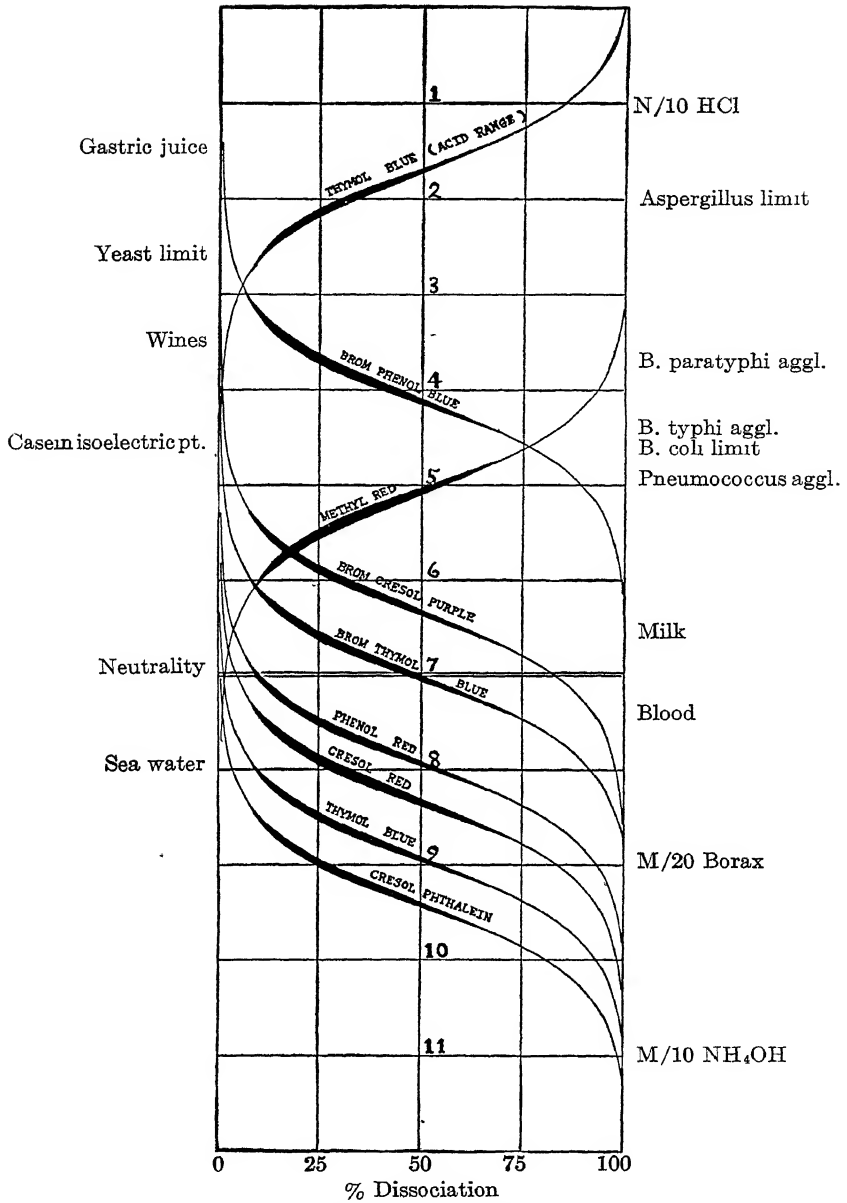


Fig 144.—Indicator curves. Shading indicates useful range. (Clark, *The Determination of Hydrogen Ions*, Williams and Wilkins Co , Publishers)

two opposing forces: the solution pressure of the metal (as the metal tends to go into solution), and the osmotic pressure of the ions in solution. Nernst has shown that the potential difference between a metal and the solution of one of its salts is given by the equation

$$E = \frac{RT}{nF} ln \frac{K}{p}$$

where E = electrode potential, F = Faraday (96,500 coulombs), *ln* = natural logarithm to base *e*, K = solution pressure of the metal, p = osmotic pressure of ions in solution, *n* = valence of metallic ion, R = gas constant (8.316 joules per degree), and T = absolute temperature.

At any given temperature, RT/F is a constant; so that E will vary with *n* and K (which depend upon the nature of the metal) and p (which depends upon the concentration of ions in solution). Using two electrodes of the *same* metal dipping into two solutions of *different* concentrations, and connecting the electrodes by wire and the solutions by a "salt bridge" (an inverted U-tube with salt solution), an electromotive force will be developed equal to the difference between the two electrode potentials; or

$$\begin{aligned} E &= E_1 - E_2 \\ &= \frac{RT}{nF} ln \frac{K_1}{p_1} - \frac{RT}{nF} ln \frac{K_2}{p_2} \\ &= \frac{RT}{nF} (lnK_1 - lnp_1 - lnK_2 + lnp_2) \end{aligned}$$

But $K_1 = K_2$, since we are dealing with the same metal. Therefore

$$E = \frac{RT}{nF} ln \frac{p_2}{p_1}$$

Now p, the osmotic pressure, is proportional to concentration, c; so that

$$E = \frac{RT}{nF} ln \frac{c_2}{c_1} \text{ (I)}$$

One type of electrode which may be used is platinum black saturated with hydrogen; this acts as a hydrogen electrode. Here such electrodes are dipped into solutions containing different concentrations of hydrogen ions. Knowing the $[H^+]$ of one solution, the $[H^+]$ of the second solution can be determined from the electromotive force produced. Let $[H^+]$ of the known solution be normal (1); then equation (I) becomes

$$E = \frac{RT}{nF} ln \frac{1}{[H^+]}$$

R, T, *n*, and F are known. The result is multiplied by 2.303 to change $\log_{ln}$ to $\log_{10}$. Then we get

$$\begin{aligned} E &= 0.058 \log \frac{1}{[H^+]} \\ \text{or } E &= 0.058 (-\log [H^+]) \\ \text{or } E &= 0.058\ pH \\ \text{or } pH &= \frac{E}{0.058} \end{aligned}$$

Often, glass, calomel and quinhydrone electrodes are used instead of the "normal" electrode; but what has been discussed illustrates the principle involved.

Colloidal Solutions.—The fact that protoplasm belongs to the group known as "colloids" makes a discussion of colloidal solutions appropriate. In a general way, the particles in suspension in a colloidal solution—where the particles do not settle—are intermediate in size between those in true solutions and coarse suspensions. The range of size of colloidal particles is approximately between 0.2 μ (or 200 mμ) and 1 mμ (where μ = 1 micron = 10^{-3} mm.; and mμ = 1 millimicron = 10^{-6} mm.). Particles larger than 200 mμ are said to be in coarse suspension and those smaller than 1 mμ are said to be in true solution.

These boundaries, chosen arbitrarily, are matters of convenience. For example, the upper limit, 200 mμ, represents the resolving power of a very good microscope with proper illumination. The lower limit, 1 mμ, is near the limit of visibility of the ultramicroscope.

The range of colloidal solutions is such that they show many properties in common with true solutions and coarse suspensions. There is a gradation of properties with increasing size—from true solution, up through colloidal solutions to the coarse suspensions; and at no place is there an abrupt break.

Graham, the "father of colloid chemistry," distinguished "crystalloids" from "colloids" on the basis of diffusion. Colloids, which are relatively large particles, diffuse slowly or not at all, while crystalloids, which are smaller particles, diffuse readily. In a practical way, this method is sometimes used to distinguish a colloidal solution from a true solution.

Colloidal solutions run through ordinary filters. The use of filter paper is a rough but convenient method for separating colloidal particles from those in coarse suspension.

To separate colloidal particles from those in true solution, filters with much finer pores than is found in filter paper must be used. These are available in membranes such as parchment, animal membranes, collodion, cellophane, etc. Using special techniques, membranes having pores of any desired size may be prepared. In this way colloidal particles can be separated from smaller ones, or even two different sizes of colloidal particles from each other. This operation is known as *dialysis* and is frequently used to purify colloidal solutions from dissolved impurities.

Colloidal solutions, in contrast to coarse suspensions, are perfectly clear under the ordinary microscope. However, to the naked eye they often appear to be turbid, especially when examined at right angles to the path of a beam of light. This is the *Tyndall effect* and is due to the fact that the particles are large enough to scatter light. In the ultramicroscope the microscope is so arranged as to look at right angles to the beam of incident light. The light is here scattered by the colloidal particles, and each particle appears as a light spot in a dark field. This enables us to examine particles too small to be seen in the ordinary microscope. In true solutions the particles are

too small even to scatter light, so that solutions appear homogeneous both in the microscope and in the ultramicroscope.

Colloidal particles in the ultramicroscope appear to be in a state of vigorous motion. It is an irregular, rapid motion and is known as the *Brownian movement*, named after Robert Brown who first observed it.

We can summarize our results thus:

Suspensions	*Colloidal solutions*	*True solutions*
Do not pass through filters	Pass through filters	Pass through filters
Do not dialyse	Do not dialyse	Do dialyse
No osmotic pressure	Little osmotic pressure	Large osmotic pressure
No depression in freezing point	Little depression in freezing point	Large depression in freezing point
No elevation in boiling point	Little elevation in boiling point	Large elevation in boiling point
Particles larger than 200 mμ	Particles between 200 mμ and 1 mμ	Particles smaller than 1 mμ
Microscopic (or larger)	Ultramicroscopic	Invisible

Surface Tension.—In the interior of a liquid the molecules are equally attracted by neighboring molecules. This is not true at the surface of a liquid, where attraction from the top is lacking. (Attraction forces due to air molecules are negligible.) Freedom of movement is, therefore, restricted at the surface, and one such result is the formation of a surface *membrane*. A certain "force" pulling the surface molecules inward is called "surface tension" (or "interfacial tension"). With colloidal particles this interfacial tension is exhibited at those points where particles and liquid meet (they meet at the "interface").

Certain types of colloids—the emulsoids, in which the colloid and the liquid medium are more or less soluble in one another—tend to decrease the surface tension of liquids. The "foaming" of such solutions is due to the lowering of the surface tension.

The temporary emulsion formed by shaking together oil and water may be converted into a permanent one by the addition of soap, which lowers the interfacial tension existing at the interface of the oil and water droplets.

Willard Gibbs was the first to show that substances which lower the surface tension tend to concentrate at the surface (they are **adsorbed**) and various properties of colloids—those dealing with precipitation, electrical charges, viscosity, etc.—are related to such "adsorption." The bearing on cellular problems becomes apparent when we remember once again that protoplasm is a colloidal system.

The extent of adsorption depends upon several factors: one such factor is the fineness of division. The finer the division—the greater the "surface"—the better the adsorption; and colloids *are* finely divided particles.

Many biological materials, usually of a protein and therefore of colloidal character, can be purified by making use of the principles of adsorption. The protein is adsorbed on some suitable material—charcoal, aluminum hydroxide, etc.—at the proper *p*H and "freed" or "eluted" at another *p*H.

REFERENCES

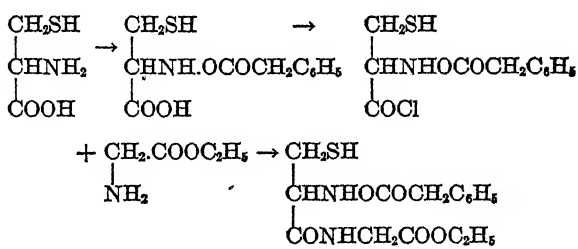
An excellent introductory physical chemistry for students of biochemistry and medicine is West, *Physical Chemistry* (1942).

Two other books, somewhat more specialized, are Johlin, *Introduction to Physical Biochemistry* (1941), and Bull, *Physical Biochemistry* (1943).

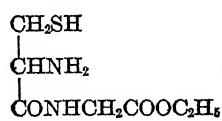
SYNTHESIS OF GLUTATHIONE (refer to p. 64)

A beautiful application of the Bergmann method is the synthesis of glutathione. Hopkins had isolated this substance from various animal tissues and from yeast, and it became evident that the compound plays a rôle in biological oxidations (see p. 400). An analysis of the compound showed it to be a tripeptide containing glycine, cysteine, and glutamic acid, but the question of how these amino acids were linked together was not settled until the glutathione was finally synthesized by Harington and by du Vigneaud. In Harington's synthesis, use is made of Bergmann's carbobenzoxy method, with the exception that the final reduction is carried out by heating (at 45° C.) with phosphonium iodide in acetic acid solution, instead of using hydrogen in the presence of platinum. The Bergmann method of reduction is impossible in the presence of cysteine derivatives due to the poisoning of the catalyst by the sulfur.

An outline of Harington's synthesis is as follows: cysteine [really cystine (p. 54), but for the sake of simplicity we use cysteine] is converted into the carbobenzoxy derivative in the way already described (p. 63) and the resulting product is converted into the acid chloride, which is then coupled with glycine ester:

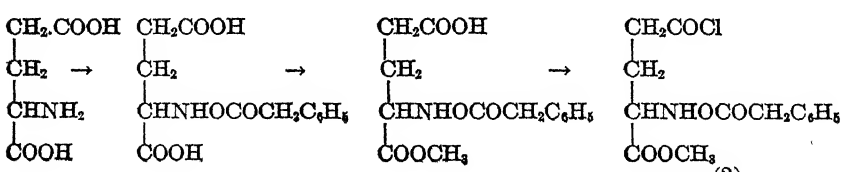


The product is treated with phosphonium iodide to remove the carbobenzoxy group (here benzyl iodide, $C_6H_5CH_2I$, is formed instead of toluene), yielding cysteylglycyl ester:



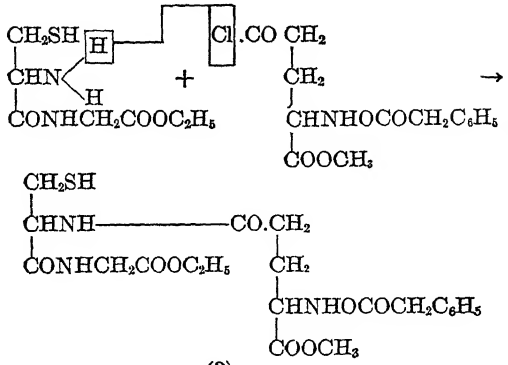
(1)

Glutamic acid is next treated with the carbobenzoxy reagent, the product converted into the α-monomethyl ester and the latter changed to the acid chloride:



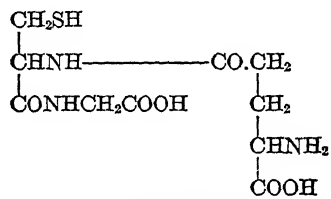
(2)

(1) and (2) are now coupled to give (3):



(3)

The ester groups are next removed from (3) by hydrolysis, and the resulting acid is treated with phosphonium iodide in the usual way, giving (4):



(4)

Glutathione.

which is glutathione, or γ-glutamylcysteylglycine.

MANUFACTURE OF NYLON (refer to p. 64)

In the manufacture of nylon adipic acid is made to combine with hexamethylenediamine:

$$HOOC(CH_2)_4COOH + H_2N(CH_2)_6NH_2 + HOOC(CH_2)_4COOH + H_2N(CH_2)_6NH_2,$$

Adipic acid. Hexamethylenediamine.

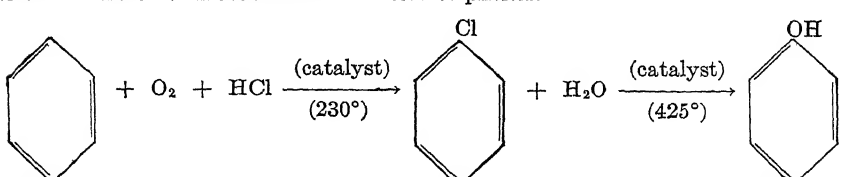
etc.

$$\rightarrow HOOC(CH_2)_4CO[NH(CH_2)_6NHCO(CH_2)_4CO]_x\ NH(CH_2)_6NH_2 + (x + 2)H_2O$$

The reaction involves the carboxyl groups of adipic acid and the amino groups of hexamethylenediamine

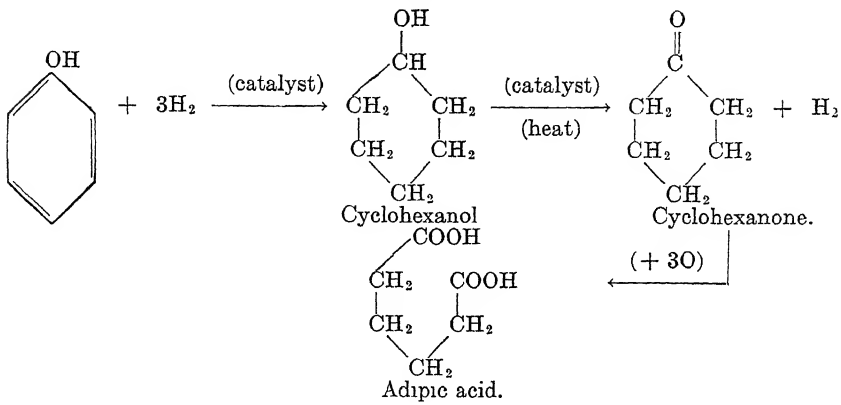
The molten product is then formed into fine filaments

Contributing to the success of the process are the methods used in preparing the raw materials. The starting point for the adipic acid is benzene, which is first converted to chlorobenzene and then to phenol:

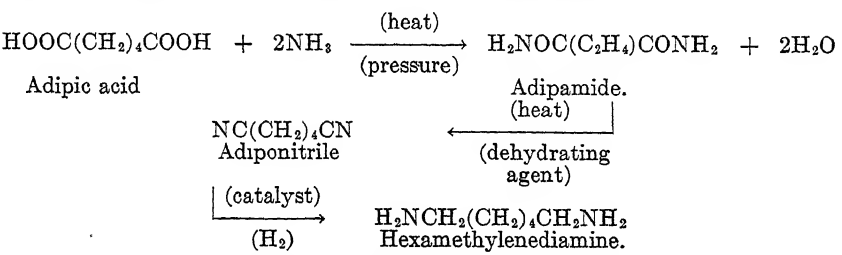


Benzene. Chlorobenzene. Phenol.

The hydrogenation of phenol converts it to cyclohexanol, which, in turn, is changed to cyclohexanone, and the latter is oxidized to adipic acid:



Part of the adipic acid is converted to hexamethylenediamine:



THE GENE AND NUCLEOPROTEINS (refer to p. 79)

Direct chemical analysis of whole chromosomes show them to be largely nucleoprotein, which suggests that genes, too, are nucleoproteins. Ultraviolet radiation induces gene mutation; and its efficiency in this respect varies with wave length in the same way as does its absorption by nucleic acid, strongly indicating that the energy effective in producing mutations in genes is absorbed by nucleic acid. The simplest assumption possible is that this is so because the nucleic acid is part of the gene. (Beadle in Green's *Currents in Biochemical Research* (1946), 5.)

PREPARATION OF CRYSTALLINE PEPSIN (refer to p. 97)

(Northrop, *Harvey Lectures*, Williams and Wilkins Co., Publishers)

Five hundred gm. Parke, Davis pepsin U. S. P. 1:10,000 dissolved in 500 ml. H_2O and 500 ml 1 normal H_2SO_4 added. One thousand ml. saturated $MgSO_4$ added with stirring. Solution filtered through fluted paper (S and S. No. 1450½) and then with suction This precipitate must not be allowed to stand at room temperature more than about twenty-four hours.

Filtrate discarded.

Precipitate 1 Wash twice with equal volume ⅔ saturated $MgSO_4$, filter with suction.

Filtrate discarded.

Precipitate 2. Stir with water to thick paste and add M/2 NaOH until complete solution. (Great care must be taken to avoid local excess of NaOH. *p*H never more than 5 0.)

M/2 H_2SO_4 added with stirring until heavy precipitate forms (*p*H about 3.0), three to six hours at 8° C., filter with suction.

Filtrate discarded.

Precipitate 3. Stir with H_2O to thick paste at 45° C , M/2 NaOH added carefully until precipitate dissolves (filter if cloudy and discard precipitate). Beaker containing filtrate placed in a vessel containing about 4 liters of H_2O at 45° C., inoculated and allowed to cool slowly, cooling should require three to four hours

and heavy crystalline precipitate should form at about 30 to 35° C. Solution kept at 20° C. for twenty-four hours. Thick crystalline paste, filter with suction.

Precipitate 4 Wash with small amount of cold H_2O and then with ½ saturated $MgSO_4$ and store under saturated $MgSO_4$ at 5° C.

Filtrate. M/2 H_2SO_4 added to *p*H 3 0, amorphous precipitate filtered off and treat as Precipitate 3.

ISOLATION OF CHYMOTRYPSIN FROM BEEF PANCREAS (refer to p. 98)

(Northrop and Kunitz)

Remove pancreas from cattle within one hour after slaughter, immerse in cold $N/4$ H_2SO_4, drain off acid, mince, suspend for twenty-four hours in 2 volumes $N/4$ H_2SO_4 at 5° C. Strain through gauze.

Acid extract of pancreas 7 liters

Add solid $(NH_4)_2SO_4$ to 0.4 saturation.
Filter

Filtrate

Add solid $(NH_4)_2SO_4$ to 0.7 saturation. Two days at 5° C.
Filter

Precipitate 100 gm.

Dissolve in H_2O. Refractionate between 0.4 and 0.7 sat. $(NH_4)_2SO_4$.
Filter

Precipitate from 0.7 sat. $(NH_4)_2SO_4$ *90 gm.*

Dissolve in 0.25 sat. $(NH_4)_2SO_4$. Adjust to *p*H 5.0, two days at 25° C.
Filter

Crystals of chymotrypsinogen 25 gm.

Recrystallize in 0.25 sat. $(NH_4)_2SO_4$ *p*H 5.0.
Filter

8 × Recrystallized chymotrypsinogen

Dissolve in $N/50$ H_2SO_4. Adjust to *p*H 7.6. Add trace of trypsin, two days at 5° C.

Solution of chymotrypsin

Adjust to *p*H 4 0. Salt out in 0.7 sat. $(NH_4)_2SO_4$.
Filter

Amorphous precipitate of chymotrypsin 5 gm.

Dissolve in $N/100$ H_2SO_4, twenty-four hours at 25° C. Filter.

Chymotrypsin crystals

Filtrate

Adjust to *p*H 4.0, precipitate in 0.7 sat. $(NH_4)_2SO_4$ and refractionate between 0.4 and 0.7 sat. $(NH_4)_2SO_4$.
Filter

Precipitate from 0.7 sat. $(NH_4)_2SO_4$ *40 gm.*

Wash with sat. $MgSO_4$. Dissolve in 0.4 M borate *p*H 9.0, cool to 5° C. Bring to 0.5 sat. $MgSO_4$, three days at 5° C.
Filter

Filtrate

Crystals of trypsinogen 10 gm.

Wash with 0.5 sat. $MgSO_4$. Dissolve in $N/50$ H_2SO_4. Bring to 0.5 sat. $MgSO_4$. Add 0.4 M borate *p*H 9.0, one day at 5° C. Filter.

Crystals of trypsin 6 gm.

GROWTH WITH HYDROLYZED PROTEIN (refer to p. 113)

The introduction of protein hydrolyates in clinical medicine has brought out the fact that such hydrolyates may not always be the equal—nutritionally speaking—of *unhydrolyzed* proteins.

Woolley has shown that some "factor" in casein (and a number of other proteins) stimulates the growth of *Lactobacillus casei;* and that this factor, even though it may be a peptide, is certainly not an amino acid. The unknown substance was called *strepogenin.* It is present in casein, trypsinogen, insulin and hemoglobin, but not in egg albumin and gelatin.

Mice and rats, fed on *hydrolyzed* casein—a protein always considered high in the biological series—did not develop normally until strepogenin (in the form of a protein rich in the substance, like casein) was added to the diet. (Editorial, *J. Am. Med. Assoc.,* **131,** 826 (1946); Woolley, *J. Biol. Chem.,* **162,** 383 (1946); Womack and Rose, *Ibid,* **162,** 735 (1946).

FOOD REQUIREMENTS FOR THE ARMY (refer to p. 135)

The American soldier is splendidly fed. There is practically no disagreement on this question. Not only does he get his three to four thousand calories per day, but careful attention is given to the biologically important foodstuffs.

But knowledge of what is biologically important is not in itself enough. If the soldier is abroad, or, still worse, within the field of active fighting, various problems arise. Much of the food may have to be imported The food may have to withstand considerable variations in climate. Problems of space and of possible spoilage require much of the food to be in a dry or dehydrated form. And a factor which must never be overlooked is palatability.

Taking such factors into consideration, several basic rations have been devised.

The first one is known as *Field Ration A.* Designed for army men in this country and, whenever practical, for men abroad, this ration is largely representative of the kind of food the average, fairly well-to-do and intelligent middle-class American family consumed before the period of civilian rationing.

Field Ration B comes into play when men are overseas and the food becomes restricted to the extent that only part of the perishable items can be supplied. The *B* ration—one of non-perishables—includes sugar, flour, salt, and canned or dehydrated meats, fruits and vegetables, largely adjusted to suit climatic conditions, and so arranged that the menus repeat themselves on a ten-day cycle—this to prevent monotony.

Both *A* and *B* rations are for garrison duty, not for actual combat. The latter requires "operational rations."

An important "operational ration" is *Field Ration C.* Here even the field kitchen is often no longer available. One such *C* ration consists of six cans. Three of these cans—one for each meal—are identical: biscuits, hard candy, soluble coffee and sugar. The remaining three cans—also one for each meal—are different. One contains meat and beans, the second meat and vegetable stew, and the third meat and vegetable hash.

A special emergency ration, *Field Ration D,* consists of three 4-ounce chocolate bars, plus some thiamine—a vitamin which helps in the metabolism of carbohydrates.

Field Ration K, a comparatively late product in the field, may replace ration *C* for combat duty. The *K* ration "consists of three separate units, one for each meal. Each is packaged in a moisture-vapor-resistant, gas resistant, non metallic container, and contains two different types of biscuits, canned meat or cheese, a confection, a beverage concentrate, chewing gum to allay thirst and four cigarets. The meat unit, confections and beverages are different for each of the three meals.

The nutritive values of these rations are given in the accompanying table.

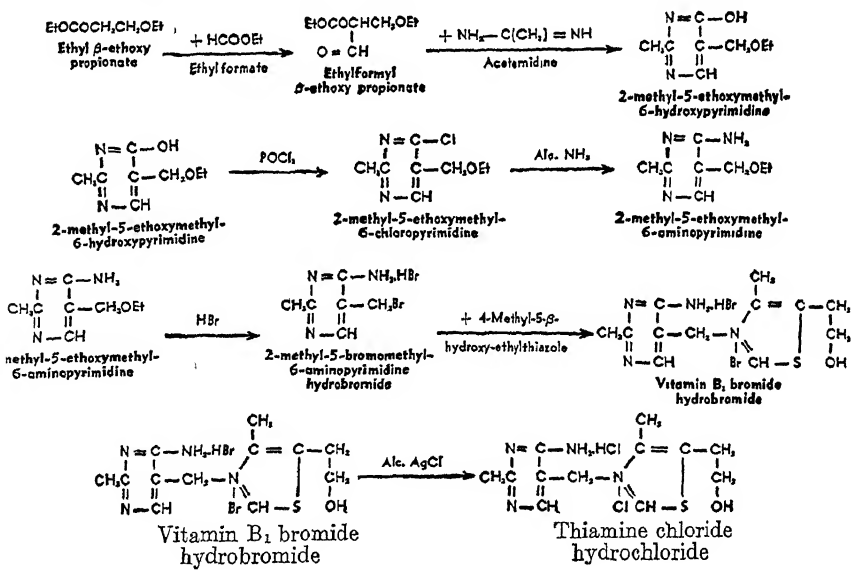
	N.R.C.*	Field ration A.	Field ration C.	Field ration K.
Calories....	4,050	4,300	2,600–3,000	3,000–3,400
Protein (gm.)	70	130	120–140	115
Fat (gm.) .	. . . .	195		
Carbohydrate (gm)	.....	510		
Calcium (mg.)	800	1,000	500	1,000
Phosphorus (mg)	..	2,000	. . . .	2,000
Iron (mg.) . .	12	25	27	22
Vitamin A, I.U	5,000	13,000	2,400	
Thiamin (mg.). .	2 15	3	1 5	3.0
Riboflavin (mg.)	3 12	2 8	1 6	2.5
Nicotinic acid (mg.)	21 5	30	26	25
Ascorbic acid (mg)	75	130	27–33	55

*N.R.C. National Research Council's recommendations for civilians. This is based on ⅔ extreme activity and ⅓ moderate activity.

(See Wodieka. *Ind. Eng. Chem.*, **35**, 12 (1943); Sebrell, *Ann. Rev. Biochem.*, **13**, 459 (1944).

SYNTHESIS OF THIAMINE (refer to p. 150)

The synthesis of the vitamin was accomplished by Williams as follows:



FOLIC ACID (refer to p. 172)

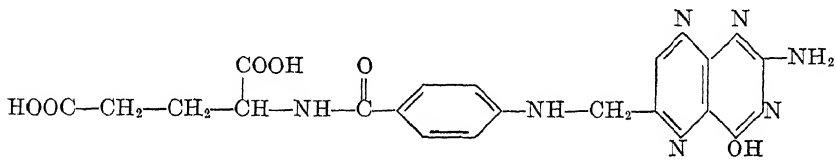
Much has been done in this field in the meantime.

The name "folic acid" is used in general to connote "the factor or factors possessing growth-stimulating properties for *Lactobacillus casei* and *Streptococcus Lactis* R, and essential for growth and hemoglobin formation in the chick."

Folic acid has anti-anemia effects in five types of anemia: Nutritional macrocytic* anemia; Addisonian pernicious anemia (p. 260); and the macrocytic anemias of sprue, pellagra and pregnancy.

The fact that both folic acid and liver extract are effective in the treatment of pernicious anemia does not mean that they are one and the same substance. In fact, the evidence points the other way: Concentrated liver extract contains little folic acid.

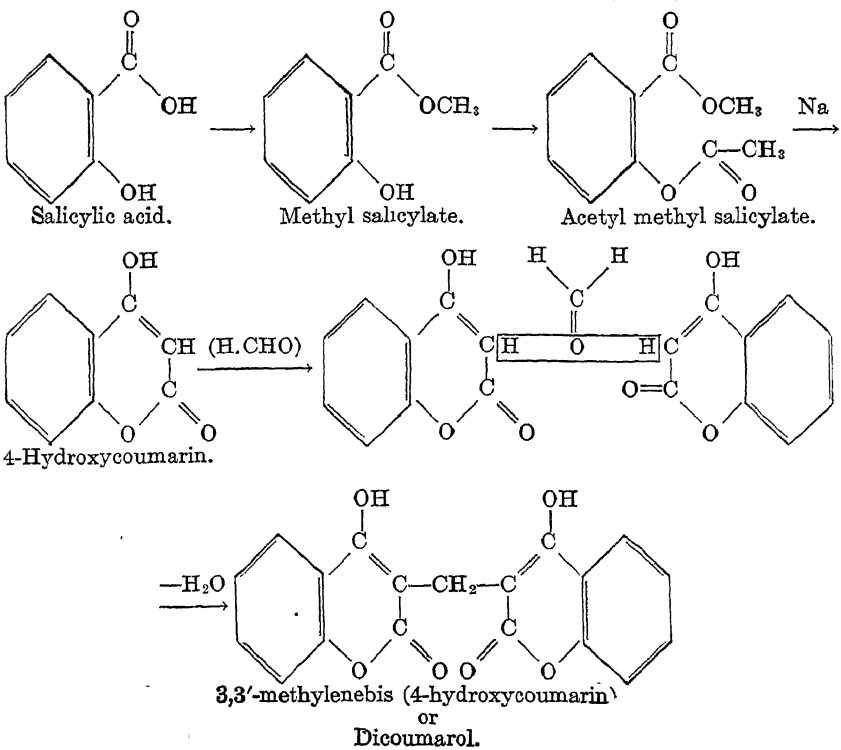
The structure of a folic acid shows it to be a pterin derivative (p. 173). The following has been assigned to it:



which shows it to contain a benzoyl nucleus as well as glutamic acid (in addition to the pteridine derivative) (*Science*, **103**, 667 (1946).

At different times "folic acid" has been given different names: Vitamin M, vitamin B_c, norit eluate factor, *Lactobacillus casei* factor, factor U, factor SLR; at least, these various compounds (including folic acid) show similar characteristics and similar biological activities. (See further, Borden's *Review of Nutritional Research*, **7**, Nos 2, 3, 4 (1946); Johnson, *J. Biol. Chem*, **163**, 255 (1946); Spies, *J. Am Med. Assoc.*, **130**, 474 (1946); *Ibid*, **130**, 496 (1946).

SYNTHESIS OF DICOUMAROL (refer to p. 271)



* Giant red blood corpuscles.

THE Rh FACTOR (refer to p. 272)

Landsteiner immunized rabbits by injections with the blood of *rhesus* monkey The serum of such rabbits agglutinized about 85 per cent of human bloods (See pp. 278, 279.) These human bloods acted as if they possessed the same immunizing or antigenic substance as the blood of the monkey; hence, the human factor was given the name rhesus or Rh factor

The bloods (red blood cells) containing the Rh factor were called Rh+ and those not containing it (about 15 per cent), Rh−. The Rh− showed no agglutination.

Landsteiner showed that the Rh factor is transmitted as a dominant trait in heredity (Root and Bailey, Jr., *Biological Review* (City College), March, 1945)

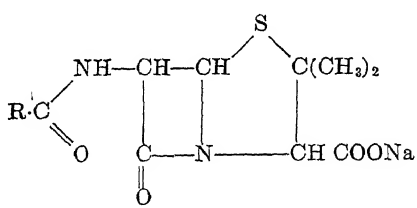
"In the normal human body the Rh antigen is harmless, and its presence is important only in child-birth, blood transfusion and paternity disputes For example, when an Rh− woman, wedded to an Rh+ man, has an Rh+ infant, there is a possibility that the Rh factor may exert its malignant influence on the mother and child This is accomplished by the flow of Rh+ antigenic substances through the placental barrier, from the fetus to the mother. In the mother's body the production of antibodies to Rh is thereby stimulated. When these antibodies return through the placenta to the fetus, they react with the blood cells of the fetus, destroying them and giving rise to a hemolytic disease of the fetus or newborn (erythroblastosis fetalis)." (*What's New* (Abbott Labs), March, 1946.)

CHEMISTRY OF PENICILLIN (refer to p. 293)

Several "types" of penicillin are known. They all have the empirical formula $C_9H_{11}O_4SN_2$ R.

In F-penicillin, R = Δ^2—pentenyl, $—CH_2\,CH = CH{\cdot}CH_2\,CH_3$.
In dihydro-F-penicillin, R is *n*-amyl.
In G-penicillin, R is benzyl.
In X-penicillin, R is *p*-hydroxybenzyl.
In K-penicillin, R is *n*-heptyl.

The pencillins are strong monobasic acids of pK about 2 8
One type of formula proposed for the penicillins is



(*Science*, **102**, 627 (1945).

FUNCTION OF INSULIN (refer to p. 316)

Cori points out that "the first step in the utilization of glucose by animal tissues, a step common to its transformation to glycogen and its oxidation, is catalyzed by hexokinase (see p. 323):

$$\text{Glucose} + \text{adenosinetriphosphate} \xrightarrow{\text{hexokinase}} \text{glucose} - 6 - \text{phosphate} + \text{adenosinediphosphate}$$

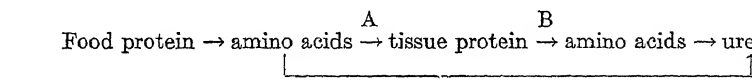
This reaction is inhibited by anterior pituitary extract, and the inhibition can be counteracted by insulin" (see p 317).

We now begin to see just what one of the functions of insulin—and a hormone from the anterior pituitary—is: It is to regulate an enzymic reaction. (Price, Cori and Colowick, *J. Biol. Chem.*, **160**, 633 (1945).

A POSSIBLE METHOD FOR TESTING THE AGING PROCESS (refer to p. 356)

The various problems relating to the aging process are well summarized by MacNider,[1] and it is not necessary to consider them here Carrel, among others, advocated the need for some specific chemical or physiological method of measuring age.[2] Such methods have been suggested from time to time. An active worker in this field has been Simms.[3] His method, applied to rats, consists in killing healthy rats in various age groups and determining the amount of bleeding required to produce death. He found that 825-day-old rats died with 12 per cent less hemorrhage than 100-day-old rats.

An entirely different method of approach has suggested itself to the author. Let us confine ourselves to a specific substance in the tissues, namely protein. The following diagram represents our present conception of the process:



At nitrogen equilibrium, protein upbuilding in the tissues is equal to protein degradation; or A = B; and, so far as we understand the process, A remains equal to B from the time nitrogen equilibrium sets in (cessation of growth) until well on in life, ceasing only in the terminal stages (assuming, of course, normal health).

But what about the *amount*[4] of A used to form tissue protein: does that at all vary with age? My postulate is that the amount of A (and therefore of B) becomes progressively smaller with age. This means that as the years roll on, less and less *new* tissue is being formed per unit of time

A method for testing this theory is at hand. For example, we may use as subjects rats from three months to two to three years of age We feed them with some suitable amino acid containing isotopic nitrogen (N*), according to the methods developed by the late Dr. Schoenheimer and his co-workers (Rittenberg, Bloch, Sheman, etc.). We then determine the amount of N* in the tissues

If the theory is correct, N* in the tissues of three-months-old rats should be higher than N* in tissues of two-year-old rats.

To put it in another way, N* taken in = (measured per unit of time) N* in tissues plus N* in urine. With time, N* in tissues becomes less, and N* in urine becomes correspondingly more. Tissue turnover—amino acid turnover—becomes less with advancing age.

Of course, all this does not tell us what *causes* such changes; but it seems to me that it gives us a possible chemical method of measuring them.

THE ORNITHINE CYCLE (refer to p. 357)

In the bread mold, *Neurospora*, Srb and Horowitz have shown the presence of the ornithine cycle similar to that postulated by Krebs for mammalian liver. (Beadle in Green's *Currents in Biochemical Research* (1946), 3.)

ALCAPTONURIA (refer to p. 364)

Beadle points out that in this disease a Mendelian trait can be interpreted in terms of specific chemical reactions. "In individuals homozygous for the mutant gene responsible for this character, homogentisic acid is excreted in the urine instead of being broken down to CO_2 and H_2O, as it is in persons receiving the normal form of alcaptonuric gene from one or both parents."

It is believed that alcaptonurics lack a specific enzyme (found in the blood of normal persons) which catalyzes the breakdown of homogentisic acid.

In alcaptonuria, it would seem, "a particular chemical reaction is controlled by a known gene through the mediation of a specific enzyme."

The failure, in certain instances, to oxidize phenylpyruvic acid (see p. 365) is also considered an example of a chemical reaction being closely connected with a specific gene.

[1] Wm. deB. MacNider, *Science*, **99**: 415, 1944.
[2] A. Carrel, *N. Y. Acad. Med.*, **45**: 1144, 1928.
[3] H. S. Simms, *Science*, **95**, 183, 1942.
[4] Or "amino acid turnover," according to Schoenheimer.

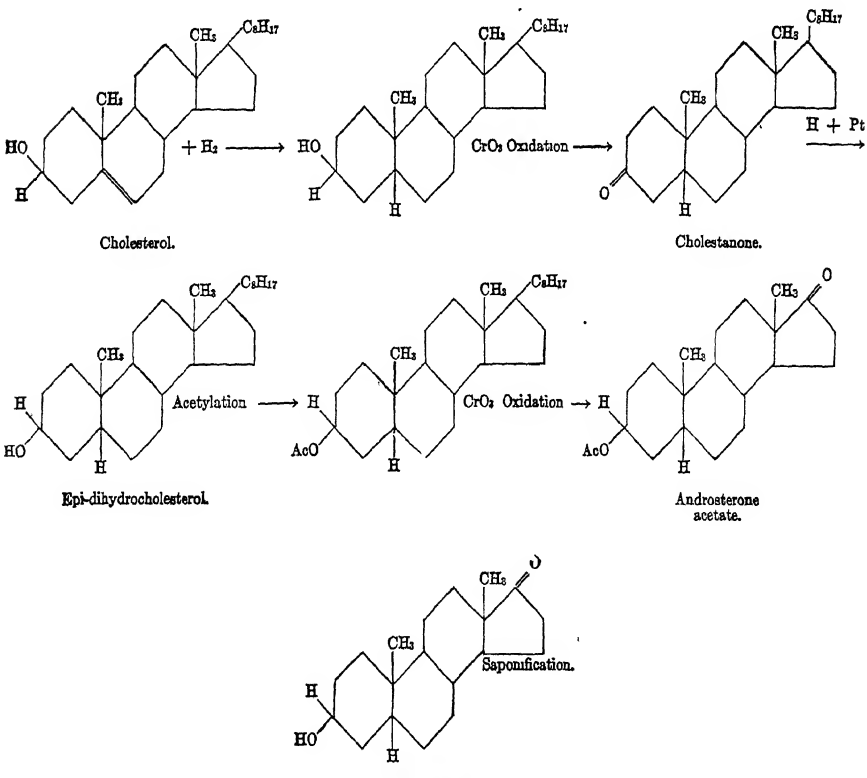
Beadle's view is that "genes act in directing specific processes." (Beadle in Green's *Currents in Biochemical Research* (1946), 1; Beadle, *Physiol. Rev*, **25**, 643 (1945); Beadle, *American Scientist*, **34**, 31 (1946).

ISOLATION OF ADRENOTROPIC HORMONE (refer to p. 491)

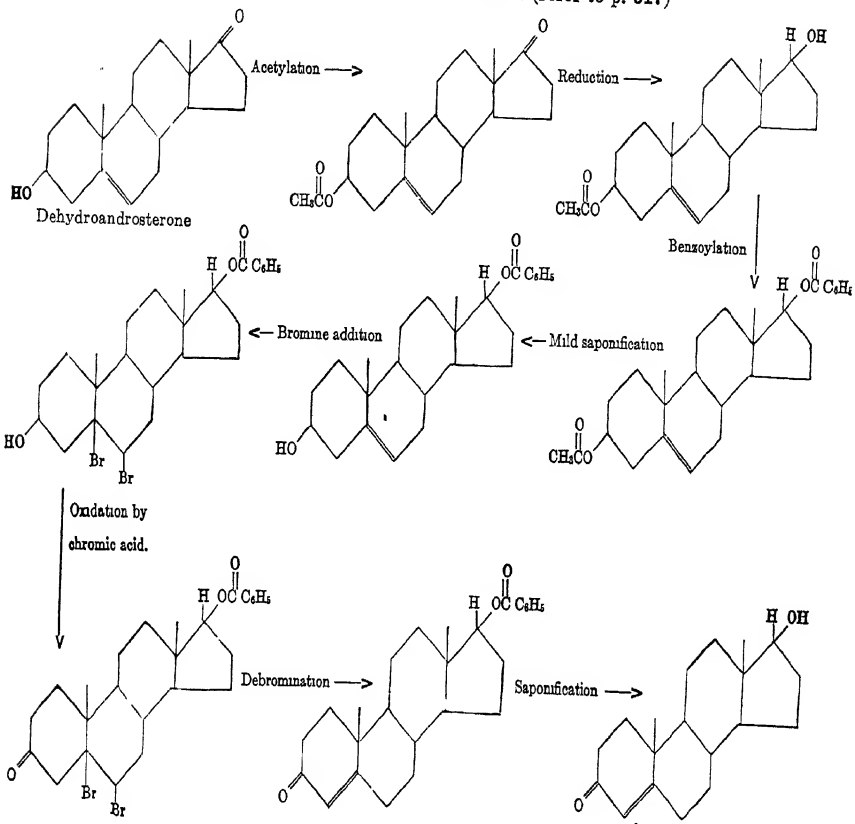
(Li, Simpson and Evans, *Science*, **96**, 450)

A method is herein described for the isolation of a protein from the anterior hypophysis which selectively stimulates the adrenal cortex and is free from other biologically active contaminants Sheep pituitaries were ground and extracted with acidified 80 per cent. acetone The extract was precipitated in 90 per cent. acetone and dried The dried powder was extracted with 0.1 M Na_2HPO_4 and the extract again precipitated by bringing it to half saturation with $(NH_4)_2SO_4$. The precipitate was then dissolved in water and dialyzed until salt-free. The dialyzed solution was adjusted to *p*H 3.0 and saturated NaCl was added to 0.54 M. The precipitate formed was saved for the isolation of lactogenic hormone and the supernatant was brought to half saturation with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitate was dissolved in water and half of its volume of concentrated NH_4OH was added and the solution allowed to stand at room temperature for 4 hours. The solution was then brought to 90 per cent. acetone. The precipitate formed was suspended in water and dialyzed, first against distilled water, then against *p*H 7.5 phosphate buffer of ionic strength 0.10. A slight precipitate that formed was discarded. Saturated aqueous $(NH_4)_2SO_4$ was then added to the dialyzed solution to 0.4 saturation. The precipitation was $(NH_4)_2SO_4$ was repeated two more times. The final precipitate was dialyzed and adjusted to *p*H 3 0 and saturated NaCl solution was added to 0.54 M. The precipitate was removed and discarded and the supernatant brought to 1.35 M. The precipitation was NaCl was repeated four times.

SYNTHESIS OF ANDROSTERONE (refer to p. 517)



SYNTHESIS OF TESTOSTERONE (refer to p. 517)



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